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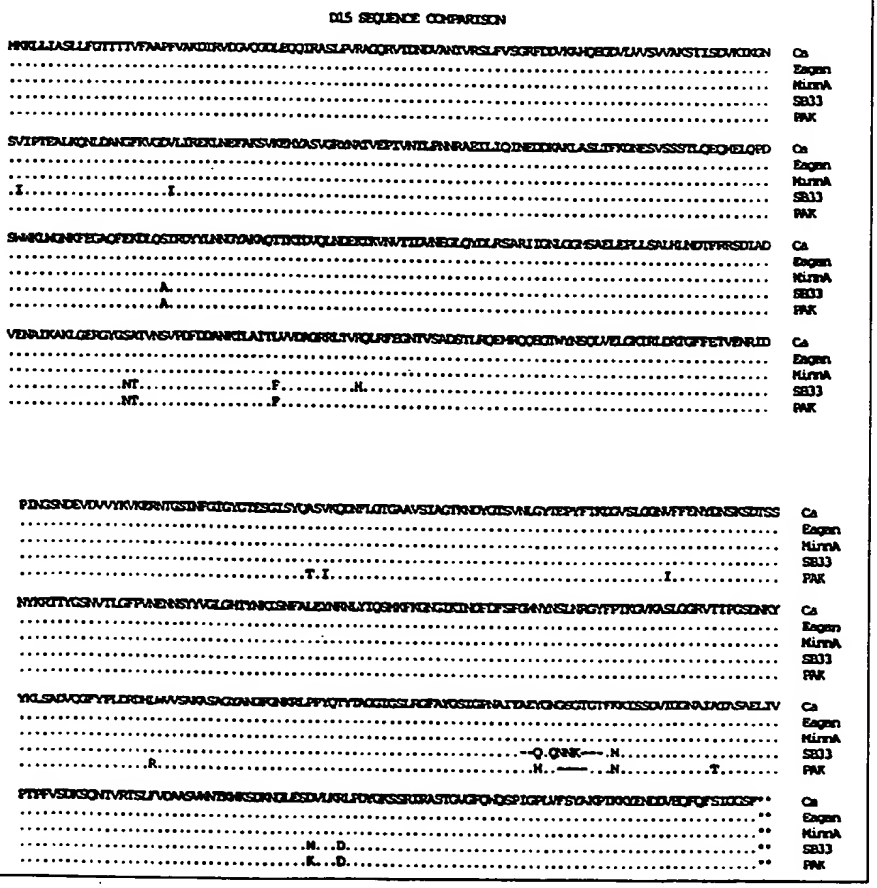
(54) Title: HAEMOPHILUS OUTER MEMBRANE PROTEIN

(57) Abstract

Purified and isolated nucleic acid from specific strains of *Haemophilus influenzae* is provided which encodes at least a portion of the D15 outer membrane protein of *Haemophilus*. The nucleic acid is used to produce peptides, polypeptides and proteins free of contaminant associated with *Haemophilus* for purposes of diagnosis and medical treatment. Furthermore, the nucleic acid may be used in the diagnosis of *Haemophilus* infection. Antisera obtained following immunization with the nucleic acid D15 outer membrane protein or peptides also may be used for the purpose of diagnosis and medical treatment.



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TITLE OF INVENTION
HAEMOPHILUS OUTER MEMBRANE PROTEIN

FIELD OF INVENTION

The present invention is related to the field of molecular genetics and is particularly concerned with the cloning of an outer membrane protein D15 of Haemophilus.

BACKGROUND OF THE INVENTION

Haemophilus influenzae type b (Hib) is a major cause of bacterial meningitis in children under the age of five years. Protective antibodies to the disease are induced by the capsular polysaccharide of the organism and a vaccine was developed that utilises the purified polyribosyl ribitol phosphate (PRP) as the antigen. This vaccine provides 90% protection in adults and in children over 24 months of age, but was ineffective in children under 24 months Zangwill et al 1993 (The references are identified in a list of reference at the end of this disclosure). Like other polysaccharide antigens, PRP does not induce the proliferation of T-helper cells, and re-immunisation fails to elicit either a booster response or an increase in memory cells. Conjugation of the PRP polysaccharide with protein carriers confers T-cell dependent characteristics to the vaccine and substantially enhances the immunologic response to the PRP antigen. Currently, there are four PRP-carrier conjugate vaccines available. These are vaccines based upon H. influenzae type b capsular polysaccharide conjugated to diphtheria toxoid, tetanus toxoid, or Neisseria meningitidis outer membrane protein (reviewed in Zangwill et al 1993).

However, the current Haemophilus conjugate vaccines only protect against meningitis caused by Haemophilus influenzae type b. They do not protect against other invasive typeable strains (types a and c) and, more importantly, against non-typeable (NTHi) strains which are a common cause of postpartum and neonatal sepsis,

pneumonia and otitis media. In the United States alone, treatment of otitis media costs between 1 and 2 billion dollars per year for antibiotics and surgical procedures, such as tonsillectomies, adenoidectomies and insertion of tympanostomy tubes. To achieve universal protection against H. influenzae related diseases in the 2 to 6 month age group and certain high risk groups, the provision of conserved, cross-reactive non-capsular H. influenzae immunogens is desirable. Methods for inducing immunity against disease are constantly improving and there is presently a move to use subunits and better defined materials as antigens. This is being undertaken to minimise or eliminate potential side-effects caused by certain native immunogens, while preserving their immunogenicity to confer protection against the disease. Therefore, it would be very attractive to develop a universal vaccine against Haemophilus using cross-reactive outer membrane proteins, fragment, analogs, and/or peptides corresponding thereto as protective antigens. Such antigens may be incorporated into the conventional H. influenzae type b conjugate vaccines as additional immunogens or used as autologous carriers for H. influenzae capsular polysaccharides. A high molecular weight outer membrane protein D15 found in non-typeable and type b stains of H. influenzae has been identified as a cross-reactive antigen (Thomas et al., 1990). D15 appears to be cell surface-exposed in its natural state and exhibits a molecular mass of about 80 kDa as judged by SDS-PAGE analysis. It would be desirable to provide the sequence of the DNA molecule that encodes this D15 outer membrane protein and peptides corresponding to portions thereof for diagnosis, immunization and the generation of diagnostic and immunological reagents. The diseases caused by Haemophilus are serious and improved methods for preventing, detecting and treating diseases such as

otitis media, epiglottitis, pneumonia, and tracheobronchitis, are required.

SUMMARY OF THE INVENTION

The present invention is directed towards the provision of purified and isolated nucleic acid molecules comprising at least a portion coding for a D15 outer membrane protein of a species of Haemophilus. The nucleic acid molecules comprising at least a portion coding for D15 outer membrane protein are useful for the specific detection of strains of Haemophilus, and for diagnosis of infection by Haemophilus. The purified and isolated nucleic acid molecules, such as DNA comprising at least a portion coding for D15 outer membrane protein, are also useful for expression of the D15 gene by recombinant DNA means for providing, in an economical manner, purified and isolated D15 outer membrane protein.

The D15 outer membrane protein or fragments thereof or analogs thereof are useful immunogenic compositions for the preparation of vaccines against diseases caused by Haemophilus, the diagnosis of infection by Haemophilus and as tools for the generation of immunological reagents. Mono- or polyclonal antisera (antibodies) raised against the D15 outer membrane protein produced in accordance with aspects of the present invention are useful for the diagnosis of infection by Haemophilus, specific detection of Haemophilus (in, for example, in vitro and in vivo assays) and for the treatment of diseases caused by infection by Haemophilus.

Peptides corresponding to portions of the D15 outer membrane protein or analogs thereof are useful immunogenic compositions for the preparation of vaccines against disease caused by Haemophilus, the diagnosis of infection by Haemophilus and as tools for the generation of immunological reagents. Mono- or polyclonal antisera raised against these peptides, produced in accordance with aspects of the present invention, are useful for the

diagnosis of infection by Haemophilus, specific detection of Haemophilus (in, for example, in vitro and in vivo assays) and for use in passive immunization as a treatment of disease caused by infection by Haemophilus.

In accordance with one aspect of the present invention, therefore, there is provided a purified and isolated nucleic acid molecule, the molecule comprising at least a portion coding for a D15 outer membrane protein. The nucleic acid molecule has a DNA sequence selected from:

(a) the DNA sequence set out in any one of Figures 1A to 1E (as described below) or its complementary strand; and

(b) DNA sequences which hybridize under stringent conditions to the DNA sequences defined in (a). The DNA sequences defined in (b) preferably has at least 90% sequence identity with the sequences defined in (a). The DNA sequence defined in (b) particularly may comprise the consensus sequence set forth in Figure 1F (as described below).

In another aspect of the present invention, there is provided a purified and isolated D15 outer membrane protein or a portion thereof. The D15 outer membrane protein may be a Haemophilus D15 outer membrane protein and more particularly an H. influenzae D15 outer membrane protein and the H. influenzae strain may be an H. influenzae type b strain, such as H. influenzae type b strains Ca or Eagan or MinnA or a non-typeable H. influenzae strain, such as PAK 12085 or SB33.

In an additional embodiment, the present invention also includes a recombinant plasmid adapted for transformation of a host, the recombinant plasmid comprising a plasmid vector into which has been inserted a DNA segment comprising the purified and isolated DNA molecule provided herein. Such recombinant plasmid comprises a plasmid vector into which a DNA segment which

comprises at least an 18 bp fragment selected from the DNA molecules as recited above is inserted. The recombinant plasmid may be plasmid DS-712-2-1 having ATCC accession number 75604, deposited November 4, 1993 and plasmid JB-1042-5-1 having ATCC accession number 75006, deposited November 4, 1993.

The plasmids may be adapted for expression of the encoded D15 outer membrane protein in a host cell, which may be a heterologous or homologous host, by incorporation into a recombinant vector, provided in accordance with a further aspect of the invention. The recombinant vector may comprise at least a DNA segment comprising at least an 18 bp fragment selected from the DNA molecules as recited above and expression means operatively coupled to the DNA segment for expression of the gene product encoded thereby in the host cell. The plasmid for expression of the encoded D15 outer membrane protein may be plasmid DS-880-1-2 having ATCC accession number 75605, deposited November 4, 1993 being adapted for expression at the D15 outer membrane protein in E. coli. The selected DNA segment may encode a polypeptide of at least 6 residues and, in particular, may be selected from those segments encoding a polypeptide of Table 2 (below). The DNA segment may further comprise a nucleic acid sequence encoding a leader sequence for export of the gene product from the host. The host for expression may be selected from, for example, Escherichia coli, Bacillus, Haemophilus, fungi, yeast or the baculovirus expression system may be used.

Additional aspects of the invention include the protein encoded by the DNA molecule comprising at least a portion coding for the D15 outer membrane protein, fragment or a functional analog of such protein, the use of the protein or analog in vaccination and diagnosis, and the generation of immunological reagents. The invention also includes antisera (antibodies) raised

against the D15 outer membrane protein encoded by the DNA molecule comprising at least a portion coding for a D15 outer membrane protein and purified peptides corresponding to portions of the D15 outer membrane protein and there are in passive immunization and treatment of diseases caused by Haemophilus.

According to another aspect of the invention, a purified and isolated peptide containing an amino acid sequence corresponding to the amino acid sequence of at least a portion of the D15 outer membrane protein or variant or mutant which retains immunogenicity. The peptide may be produced by recombinant methods or peptide synthesis whereby the purified peptide is free from contaminants associated with bacteria normally containing the D15 outer membrane protein. Such synthetic peptides preferably have an amino acid sequence selected from those presented in Table 2.

In accordance with an additional aspect of the invention, an immunogenic composition is provided which comprises the D15 outer membrane protein, fragments thereof, functional analogs thereof, or peptides as recited above and a physiologically-acceptable carrier therefor. Such immunogenic composition is particularly formulated as a vaccine for in vivo administration to protect against diseases caused by Haemophilus. For such purpose, the immunogenic composition may be formulated as a microparticle preparation, capsule preparation or liposome preparation. In addition, such immunogenic composition may be provided in combination with a targeting molecule for delivery to specific cells of the immune system or to mucosal surfaces.

In accordance with a further aspect of the invention, there is provided a method for inducing protection against disease caused by Haemophilus, comprising the step of administering to a subject, including a mammal, such as a human, an effective amount

of the immunogenic composition or the nucleic acid molecule as recited above to provide protective immunity against Haemophilus infection.

The present invention further includes a chimeric molecule comprising a D15 protein or peptide corresponding thereto as provided herein linked to another polypeptide or protein or a polysaccharide. The linked polypeptide or protein may comprise a surface protein or peptide corresponding thereto from a pathogenic bacteria, which may be the P1, P2 or P6 outer membrane protein of H. influenzae. The linked polysaccharide preferably comprise a PRP molecule from H. influenzae.

BRIEF DESCRIPTION OF THE FIGURES

The present invention will be further understood from the following description with reference to the drawings, in which:

Figure 1A shows the nucleotide sequence of the D15 gene from H. influenzae type b Ca strain (SEQ ID NO: 1) and its deduced amino acid sequence (SEQ ID NO: 2);

Figure 1B shows the nucleotide sequence of the D15 gene from H. influenzae type b Eagan strain (SEQ ID NO. 3) and its deduced amino acid sequence (SEQ ID NO: 4);

Figure 1C shows the nucleotide sequence of the D15 gene from H. influenzae type b MinnA strain (SEQ ID NO. 5) and its deduced amino acid sequence (SEQ ID NO: 6);

Figure 1D shows the nucleotide sequence of the D15 gene from H. influenzae non-typeable SB33 (SEQ ID NO. 7) and its deduced amino acid sequence (SEQ ID NO: 8);

Figure 1E shows the nucleotide sequence of the D15 gene from H. influenzae non-typeable PAK 12085 (SEQ ID NO. 9) and its deduced amino acid sequence (SEQ ID NO: 10);

Figure 1F shows an alignment of the nucleotide sequences of the D15 genes (SEQ ID NOS: 1, 3, 5, 7 and 9)

obtained from different H. influenzae isolates (typeable, Ca, Eagan and MinnA; nontypeable SB33 and PAK 12085);

Figure 2 shows restriction maps of clones pUC19/D15 (Ca), DS-712-2-1 (Eagan), DS-691-1-5 (MinnA), JB-1042-5-1 (SB33), and JB-1042-9-4 (PAK 12085). H = HindIII; R = EcoRI; S = Sau3A I; and Xb = XbaI;

Figure 3 shows an alignment of the amino acid sequences of D15 outer membrane proteins (SEQ ID NOS: 2, 4, 6, 8 and 10) obtained from different H. influenzae isolates (typeable, Ca, Eagan and MinnA; nontypeable, SB33 and PAK 12085). Amino acids are represented by the conventional one-letter code. The Ca D15 sequence is used as reference and the dots indicate amino acid residues which are identical to those of the Ca D15 outer membrane protein;

Figure 4 shows the construction of a plasmid (DS-880-1-2) expressing full-length SB33 D15 (rD15) from the strong inducible T7 promoter;

Figure 5 shows an SDS-PAGE analysis of native D15 affinity-purified from H. influenzae strain 30;

Figure 6 shows an SDS-PAGE analysis of sequential fractions obtained during the purification of the full-length rD15 expressed in E. coli containing plasmid DS-880-1-2;

Figure 7 shows guinea pig IgG antibody responses to full length rD15. The arrows indicate the immunization schedule. Bleeds were taken at 0, 2, 4, 6 and 8 weeks. The bars represent the standard deviation;

Figure 8 shows mouse IgG antibody responses to full length rD15. The arrows indicate the immunization schedule. Bleeds were taken at 0, 1, 4, 5 and 7 weeks. The bars represent the standard deviation;

Figure 9 shows an SDS-PAGE analysis of the N-terminal rD15 fragment purified from GST-(D15 fragment) fusion protein. Lanes: 1, prestained low molecular weight markers (14kDa, 21kDa, 31 kDa, 45kDa, 68kDa,

97kDa); 2, GST standard; 3, GST-(D15 fragment) fusion protein; 4, fusion protein cleaved by thrombin; 5, N-terminal rD15 fragment; 6, GST; 7, low molecular weight markers;

Figure 10 shows guinea pig IgG antibody response to N-terminal rD15 fragment. The arrows indicate the immunization schedule. Bleeds were taken at 2, 4, 6 and 8 weeks. The bars represent the standard deviation; and

Figure 11 shows the hydrophilicity plot of D15 established by using a window average across 7 residues according to Hope, 1986.

GENERAL DESCRIPTION OF THE INVENTION

Any Haemophilus strains that have D15 genes may be conveniently used to provide the purified and isolated nucleic acid molecules (which may be in the form of DNA molecules), comprising at least a portion coding for a D15 outer membrane protein as typified by embodiments of the present invention. Such strains are generally available from clinical sources and from bacterial culture collections, such as the American Type Culture Collection. H. influenzae strains may include types a, b and c strains, non-typeable strains and other bacteria that produce a D15 protein, fragment or analog thereof. Appropriate strains of Haemophilus include:-

H. influenzae type b strain Ca;
H. influenzae type b strain MinnA;
H. influenzae type b strain Egan;
H. influenzae non-typeable b strain SB33; or
H. influenzae non-typeable b strain PAK 12085.

In this application, the term D15 outer membrane protein is used to define a family of D15 proteins which includes those having naturally occurring variations in their amino acid sequences as found in various strains of, for example, Haemophilus. The purified and isolated DNA molecules comprising at least a portion coding for D15 outer membrane protein of the present invention also

include those having naturally occuring variations in their nucleic acid sequences as found in various strains of, for example Haemophilus and those DNA molecules encoding functional analogs of D15 outer membrane protein. In this application, a first protein is a functional analog of a second protein if the first protein is immunologically related with and/or has the same function as the second protein. The functional analog may be, for example, a fragment of the protein or a substitution, addition or deletion mutant thereof.

In aspects of the present invention, the D15 gene was isolated from H. influenzae type b strain Ca as shown in Figure 1A; H. influenzae type B Eagan, Figure 1B; H. influenzae type b MinnA, Figure 1C; non-typeable H. influenzae SB33, Figure 1D; non-typeable H. influenzae PAK 12085, Figure 1E. A comparison of the nucleic acid sequences of the D15 genes and of the deduced amino acid sequences of the D15 outer membrane proteins from these strains of H. influenzae showed the genes and proteins to be highly conserved (Figures 1F and 3). The consensus sequence (SEQ ID NO: 55) for the D15 gene is shown in Figure 1F.

The purified and isolated DNA molecules comprising at least a portion coding for a D15 outer membrane protein of a species of Haemophilus, typified by the embodiments described herein, are advantageous as:

- nucleic acid probes for the specific identification of Haemophilus strains in vitro or in vivo;

- the products encoded by the DNA molecules are useful as diagnostic reagents, antigens for the production of Haemophilus-specific antisera, for vaccination against the diseases caused by species of Haemophilus and detecting infection by Haemophilus; and

- peptides corresponding to portions of the D15 outer membrane protein as typified by the embodiments

described herein are advantageous as diagnostic reagents, antigens for the production of Haemophilus-specific antisera, for vaccination against the diseases caused by species of Haemophilus and for detecting infection by Haemophilus.

Reference will now be made in detail to the presently preferred embodiments of the invention, which together with the following Examples, serve to explain the principle of the invention. For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following sections:

(i) The DNA sequences coding for the outer membrane protein D15 from H. influenzae type b Ca strain.

A clone producing the outer membrane protein designated D15 of H. influenzae type b (Hib) was isolated by screening a genomic library with H. influenzae type b OMP-specific polyclonal antibodies as previously described by Berns and Thomas 1965; Thomas and Rossi 1986. The DNA fragment encoding the D15 protein was isolated, subcloned into pUC19 to produce pUC19/D15 (Figure 2) and used to transform E. coli HB101 as described in Example 1. Plasmid DNA was prepared from two individual colonies of E. coli HB101 containing the pUC19/D15 plasmid. Sequencing was performed on an ABI DNA sequencer model 370A using dye-terminator chemistry and oligonucleotide primers which had been synthesized on an ABI DNA synthesizer model 380B, and purified by chromatography. Nucleotide sequence analysis of the D15 gene revealed that it contains a putative promoter and an open reading frame encoding 789 amino acids (Figure 1A).

The first 19 amino acid residues of the translated open reading frame form a typical leader sequence as found in other H. influenzae type b outer membrane proteins, such as P1 and P2. The N-terminal sequence of immuno-affinity purified native D15 antigen was

determined by automated Edman degradation using the ABI 477A protein sequencer and was found to be Ala-Pro-Phe, which is identical to the N-terminal amino acid sequence Ala-Pro-Phe-Val-Ala-Lys- (SEQ ID NO: 11) predicted from an analysis of the sequence of the D15 gene presented in Figure 1A.

(ii) The sequence of D15 genes from other H. influenzae strains.

D15 genes were isolated from other H. influenzae strains by screening the chromosomal libraries of H. influenzae type b strains Eagan, Minn A and the non-typeable H. influenzae (NTHi) strains SB33 and PAK 12085, as described in Examples 2, 3 and 4. Hybridization-positive clones were plated and submitted to a second round of screening. The restriction maps of the clones obtained are shown in Figure 2. The nucleotide sequences of the D15 genes were determined for all these clones (Figures 1B to 1E) and their derived amino acid sequences compared (Figure 3). The D15 amino acid sequences of the three H. influenzae type b strains were identical and only a few amino acid differences were observed in the amino acid sequence of the D15 protein from the non-typeable strains (Figure 3).

(iii) Expression of D15 and its fragments in E. coli.

Since D15 is expressed in small quantities by strains of H. influenzae, it is advantageous to either express this antigen as a recombinant protein in a heterologous system, such as E. coli, or to modify the H. influenzae organism to enhance native D15 expression. The Hind III/Eco RI fragment of H. influenzae type b Ca strain DNA encoding the full length D15 protein was expressed in pUC19 but not pUC18, suggesting that the lac promoter is helping to express the D15 gene in E. coli, even though the native D15 gene promoter is present. The T7 expression system is a tightly controlled, inducible system which has great utility in expression of

heterologous proteins in E. coli. The T7 expression system is described in U.S. Patent 4,952,496. Clones were, therefore, constructed which utilize the T7 system to express a mature D15 protein that contains an additional methionine residue at the amino terminus. The D15 signal sequence was removed during this construction process. A full length recombinant D15 (termed rD15) was expressed in inclusion bodies which allow the D15 protein to be readily purified. The D15 genes from H. influenzae type b strain Ca and H. influenzae non-typeable SB33 strain have been expressed at high levels in E. coli using the T7 system to permit production of large quantities of rD15 protein. The construction of clone DS-880-1-2 which expresses the SB33 D15 gene is described herein (see Figure 4 and Example 5). The rD15 protein was immunologically similar to its native counterpart isolated from H. influenzae typeable and non-typeable strains (see below). Thus, rD15 may be used as a cross-reactive antigen in a diagnostic kit to detect many, if not all, strains of H. influenzae and other bacteria that produce a D15 outer membrane protein or analog thereof. Alternatively, rD15 can be used as an antigen to specifically detect the presence of H. influenzae in a sample.

A truncated D15 fragment was expressed in E. coli as a fusion protein with glutathione S-transferase (GST), as described in Example 6. The construction was designed to express the N-terminal fragment of the D15 protein. The fusion protein was expressed at high levels from a pGEX-2T construction and the N-terminal fragment was cleaved from the GST carrier protein by treatment with thrombin. This procedure generated a molecule termed the N-terminal rD15 fragment which encompasses amino acids 63-223 of the D15 protein. This N-terminal rD15 fragment was highly immunogenic and elicited protective antibodies against challenge with live H. influenzae.

(iv) Purification of native D15 from H. influenzae cell paste.

The present invention also provides a method to prepare purified native D15 protein from H. influenzae. The protein is extracted and affinity-purified from the cell pastes of either H. influenzae typeable or non-typeable isolates by a procedure involving the dissolution of the protein in an aqueous detergent solution (see Example 13). The native D15 protein from a non-typeable H. influenzae strain 30 was solubilized with a 50 mM Tris-HCl/ 0.5% Triton X-100/ 10 mM EDTA buffer, pH 8.0 and further purified on a D15-specific monoclonal antibody affinity column (Figure 5A). An 80 kDa protein was eluted from the column with 50 mM diethylamine, pH 12.0 and shown to react with a D15-specific monoclonal antibody on immunoblot analysis (Fig. 5B). The native D15 is also highly immunogenic in experimental animals. Rabbit anti-D15 antisera reacted with all H. influenzae isolates as determined by immunoblot analyses.

(v) Purification of a full-length recombinant D15 protein expressed in E. coli.

A full-length recombinant D15 (rD15) protein was expressed in inclusion bodies in E. coli. As shown in Figure 6, purification of rD15 inclusion bodies was achieved by a sequential extraction of the E. coli cell lysate with 50 mM Tris-HCl, pH 8.0, then 50 mM Tris containing 0.5% Triton X-100 and 10 mM EDTA, pH 8.0. After centrifugation, more than 95% of the proteins in the resulting pellet was an 80 kDa protein by SDS-PAGE analysis, that reacted with a D15-specific monoclonal antibody on an immunoblot. The N-terminal sequence of the rD15 was found to be Met-Ala-Pro-Phe-Val-Lys-Asp- (SEQ ID NO: 54) which is identical to the predicted amino acid sequence.

The rD15 inclusion bodies were solubilized with a mixture of PBS, 0.5% Triton X-100, 10 mM EDTA and 8 M

urea (see Example 8). After dialysis against PBS to remove urea, more than 80% of the D15 protein remained soluble. This soluble rD15 antigen was used for the immunogenicity studies described below. From shake-flask experiments, it was estimated that about 10 mg of soluble rD15 protein was obtained from 1 L of E. coli bacterial culture. It is clear that growing the recombinant E. coli strains under optimised fermentation conditions significantly increase the level of rD15 production.

(vi) Immunogenicity of the full-length recombinant D15 protien (rD15).

The immunogenicity of the full-length rD15 protein was studied in guinea pigs and mice. Using the immunization protocols described in Figure 7, a 15 μg dose of rD15 induced high IgG titers in guinea pigs when administered in the presence of either Freund's adjuvant or $AlPO_4$. In the mouse dose-response study, the protein appeared to be immunogenic at a dose as low as 5 μg in either Freund's adjuvant (Figure 8A) or $AlPO_4$ (Figure 8B).

The protective ability of rD15 against H. influenzae type b infection was examined in the infant rat model of bacteremia essentially as described by Loeb (1987). Thus, infant rats passively immunized with guinea pig anti-rD15 antisera were significantly less bacteremic than controls injected with pre-bleed sera, which is consistent with the previous report by Thomas et al. (1990).

(vii) Purification and characterization of the N-terminal rD15 fragment.

The truncated rD15 fragment corresponding to the N-terminus of the D15 protein (residues 22 to 223) as described in Example 6, was expressed in E. coli as a soluble protein fused to GST. The fusion protein (46 kDa) was readily extracted using phosphate buffered saline (PBS). Purification of the GST-D15 fragment fusion

protein was achieved by a single-step affinity purification process on a glutathione-Sepharose 4B column (Figure 9, Lane 3). Cleavage of the 46 kDa fusion protein with thrombin yielded two fragments (Figure 9, Lane 4), a 26 kDa protein which corresponded to a purified GST standard (Figure 9, Lane 2), and a 20 kDa polypeptide which had the size expected for the N-terminal rD15 fragment (amino acid residues 63 to 223), respectively. Separation of these two proteins was achieved by a second round of glutathione-Sepharose 4B affinity chromatography. From shake-flask experiments, it was estimated that about 1 mg of purified N-terminal rD15 fragment was recovered from 1 L of E. coli bacterial culture. It is clear that growing the recombinant E. coli strains under optimised fermentation conditions will significantly increase the level of N-terminal rD15 fragment production.

The identity of the 20 kDa polypeptide and the 26 kDa protein was confirmed by both immunoblotting and protein sequencing. The N-terminal sequence of the 20 kDa polypeptide was found to be NH_2-Ser-Leu-Phe-Val-Ser-Gly-Arg-Phe-Asp-Asp-Val-Lys-Ala-His-Gln-Glu-Gly-Asp-Val-Leu-Val-Val-Ser- (SEQ ID NO: 12), which corresponds to residues 63 to 85 of the primary sequence of D15. This result indicates that there is a spurious thrombin cleavage site within the D15 sequence and that the first 42 amino acids of the rD15 fragment are cleaved off during thrombin digestion. Thus, the final N-terminal rD15 fragment was 161 amino acids in length corresponding to residues 63 to 223 of the primary sequence of D15. The N-terminal sequence obtained for the 26 kDa protein (NH_2-Met-Ser-Pro-Ile-Leu-Gly-Tyr-Trp-Lys- - SEQ ID NO: 13) confirmed that it was GST.

(viii) Immunogenicity of the N-terminal rD15 fragment.

The immunogenicity of the N-terminal rD15 fragment was tested in guinea pigs using various adjuvants. Using

the immunization protocols described in Figure 10, a 10 μg dose of N-terminal rD15 fragment induced a good booster response in guinea pigs with almost all the adjuvants tested. The highest anti-D15 IgG titer was observed in the group of guinea pigs immunized with N-terminal rD15 fragment in Freund's adjuvant. The second best adjuvant was Titermax (CytRx Inc.). The other two adjuvants, TPAD4 (tripalmityl-Cys-Ser-Glu_4) and $AlPO_4$ were equally potent.

(ix) Protective ability of the N-terminal rD15 fragment against H. influenzae type b challenge.

An in vivo challenge model for a assessing the protective abilities of antigen against diseases caused by Haemophilus is the infant rat model of bacteremia as described by Loeb 1987. The protective ability of the N-terminal rD15 fragment against H. influenzae type b challenge was examined in this rat model. As illustrated in Table 1, infant rats passively immunized with rabbit anti-N-terminal rD15 fragment antisera showed significantly lower bacteremia compared to those injected with pre-bleed sera.

Since passively transferred antisera against the N-terminal rD15 fragment were found to be protective in the infant rat model of bacteremia, it was of interest to identify the protective epitope(s) of this N-terminal rD15 fragment. The first nine overlapping peptides of the D15 protein as listed in Table 2 were chemically synthesized based upon the amino acid sequence derived from the sequence of the D15 gene from H. influenzae type b Ca (Figure 1). These synthetic peptides were assessed for their reactivities with either rabbit or guinea pig antisera raised against purified N-terminal rD15 fragment by ELISAs. As shown in Table 3, both guinea pig and rabbit antisera reacted with a cluster of D15 peptides, including peptides D15-P4 to D15-P8 encompassing residues 93 to 209 of the D15 primary sequence.

Further studies were performed to determine whether the protection against H. influenzae type b observed using rabbit anti-D15 antisera in infant rats could be neutralized by D15 peptides. In the first experiment, a rabbit anti-N-terminal rD15 fragment antiserum was injected into a group of seven infant rats in the presence or absence of a mixture of the nine D15 peptides (D15-P2 to D15-P10). Animals in the positive control group were injected with the rabbit anti-N-terminal rD15 fragment antiserum mixed with purified D15 fragment and the negative control group was injected with a mixture of the nine peptides only. As illustrated in Table 4, infant rats passively immunized with a rabbit anti-N-terminal rD15 fragment antiserum (group #1) showed a significantly lower bacteremia level (3%, $p = 1.2 \times 10^{-7}$) compared to those in the negative control group (group #4, 100%), which was consistent with the previously obtained results. The protection mediated by the rabbit anti-N-terminal rD15 fragment antiserum was largely neutralized by the addition of purified N-terminal rD15 fragment (group #3, 64%), as indicated by the lack of significant difference in the bacteremia level between group #3 and group #4 ($p = 0.09$). Although the addition of the mixture of nine D15 peptides only slightly neutralized the protection conferred by the antiserum (group #2, 13%) as compared to group #1 (3%), the difference in bacteria counts between these two groups was statistically significant ($p = 0.0037$).

To more clearly define the protective epitope(s) of the N-terminal rD15 fragment, the above experiment was repeated with a mixture of five peptides (peptides D15-P4 to D15-P8) which were chosen for their strong reactivities with the rabbit anti-N-terminal rD15 fragment antiserum. The results obtained from this second experiment showed that the protection observed using rabbit anti-N-terminal rD15 fragment (Table 5, group #1)

was completely blocked by the addition of this mixture of five peptides (Table 5, group #2, 106%, $p = 0.53 \times 10^{-8}$). These results strongly indicate that a cocktail of D15 synthetic peptides may be used as immunogens to induce protective antibodies against H. influenzae.

(x) Epitope prediction and peptide synthesis.

To map the immunodominant T-cell or B-cell epitopes of D15, overlapping synthetic peptides covering the entire D15 protein sequence (Table 2 - SEQ ID NO: 14 to 49) were synthesized using the t-Boc solid-phase peptide synthesis as described in Example 15. The peptides were chosen based on their high index of hydrophillic β-turns estimated by secondary structure prediction analysis (Figure 11). Such peptides are likely to be surface-exposed and antigenic. Peptides more than 25 residues in length were selected to better mimic native epitopes.

(xi) Identification and characterization of immunodominant epitopes of D15 using synthetic peptides.

To map the linear B-cell epitopes of D15, overlapping synthetic peptides representing the entire sequence of D15 were individually coated onto ELISA plates and probed with several anti-rD15 antisera as described in Example 19. The results are summarized in Table 6. Mouse antisera raised against rD15 reacted with all D15 peptides, but the major epitopes were located within peptides D15-P8 (residues 180-209 - SEQ ID NO: 21), D15-P10 (residues 219-249 - SEQ ID NO: 23), D15-P11 (residues 241-270 - SEQ ID NO: 24), and D15-P26 (residues 554-582 - SEQ ID NO: 39), respectively. Rabbit anti-D15 antisera recognized only peptides D15-P4 (residues 93-122 - SEQ ID NO: 17), D15-P14 (residues 304-333 - SEQ ID NO: 27) and D15-P36 (residues 769-798 - SEQ ID NO: 49). Guinea pig antisera raised against rD15 reacted with peptides D15-P2 (residues 45-72 - SEQ ID NO: 15), D15-P4 (residues 93-122 - SEQ ID NO: 17), D15-P6 (residues 135-164 - SEQ ID NO: 19), D15-P8 (residues 180-209 - SEQ ID

NO: 21), D15-P14 (residues 304-333 - SEQ ID NO: 27), D15-P27 (residues 577-602 - SEQ ID NO: 40). The immunodominant linear B-cell epitopes of D15 were thus found to be located within peptides D15-P4 (residues 93-122 - SEQ ID NO: 17) and D15-P14 (residues 304-333 - SEQ ID NO: 27), since these are the only two peptides recognized by rD15-specific antisera from all three animal species. These results indicate that the peptides containing the linear B-cell epitope sequences described above can be used as target antigens in, for example, diagnostic kits to detect the presence of anti-D15 and anti-H. influenzae antibodies in samples.

(xii) Identification and characterization of immunodominant T-cell epitopes of D15 using synthetic peptides.

The importance of cytokine networks in the immune and inflammatory responses in immunity and inflammation and their alteration in pathology is becoming more evident as new members of the cytokine family are identified and characterized. Mills et al. (1993) have recently reported that there is a rapid clearance of B. pertussis from the lungs of mice on challenge six weeks after respiratory infection or following two immunizations with the whole-cell pertussis vaccine. Spleen cells from these immunized mice were found to secrete high levels of IL-2 and IFN-γ and low levels of IL-5 in the presence of pertussis antigen (pertussis toxoid, filamentous haemagglutinin (FHA) and pertactin). This result suggests that Th1 cell (T-cells producing high levels of IL-2 and IFN-γ) proliferation is very important for recovering from respiratory infection. The generation of Th1 and Th2 cell subsets is regulated by the balance between different groups of cytokines, predominantly IL-12 and IL-4 (Trinchieri, 1993). IL-12 and IL-4 are responsible for Th1 and Th2 cells differentiation, respectively. One of the roles of Th2

cells in the immune system is to provide helper activity for eliciting high levels of antigen-specific antibodies following immunization. Antigens containing Th1 epitope(s) stimulate antigen-specific T-cells to produce high levels of IL-2 and IFN-γ, whereas Th2 epitope(s) induce high levels of IL-4 expression. Th0 epitope(s) stimulate the synthesis of IFN-γ and IL-4.

Little is known about the cellular immune response to outer membrane proteins of H. influenzae and its role in the protection against H. influenzae infection and diseases. To this end, the inventors performed studies of the cellular response elicited in mice following rD15 immunization. D15-specific T-cell epitopes were determined using D15 peptides and T-cell lines obtained from five BALB/c mice immunized with rD15 (see Example 23). The lymphocyte proliferative responses of the D15-specific T-cell lines to overlapping D15 peptides were determined in conventional cytokine assays as described in Example 24. The results summarized in Table 7, revealed that stimulation only with certain synthetic peptides elicited proliferative responses and the release of specific cytokines. Synthetic peptides corresponding to residues 114-143 (D15-P5 - SEQ ID NO: 18), 282-312 (D15-P13 - SEQ ID NO: 26) and 577-602 (D15-P27 - SEQ ID NO: 40), and 219-249 (D15-P10 - SEQ ID NO: 23), 262-291 (D15-P12 - SEQ ID NO: 25), 390-416 (D15-P18 - SEQ ID NO: 31), 410-435 (D15-P19 - SEQ ID NO: 32) 554-582 (D15-P26 - SEQ ID NO: 39), 596-625 (D15-P28 - SEQ ID NO: 41), 725-750 (D15-P34 - SEQ ID NO: 47) and 745-771 (D15-P35 - SEQ ID NO: 48) were shown to be highly stimulatory for rD15-specific BALB/c Th0 cells and Th1 cells, respectively. Therefore, these immunodominant T-cell epitopes can be used as autologous carriers for PRP, and/or OMP B-cell epitopes to enhance their immunogenicity. The Th1 cell epitopes identified above may be useful in the H.

influenzae vaccine formulations to induce H. influenzae-specific cellular immune responses.

(xiii) Immunogenicity of D15 peptides.

To determine whether synthetic D15 peptides were immunogenic free peptides were assessed individually for their immunogenicity. Rabbit and guinea pig anti-peptide antisera were tested for their reactivities with the immunizing peptides as well as with native D15 and rD15 by ELISA and immunoblotting. As shown in Table 8, all guinea pig anti-D15 peptide antisera except those raised against D15-P26 (SEQ ID NO: 39), D15-P29 (SEQ ID NO: 42), D15-P30 (SEQ ID NO: 43) and D15-P31 (SEQ ID NO: 44) were shown to be immunogenic by ELISAs. The induction of high titers of peptide-specific IgG antibodies by free peptides clearly indicates that most peptides contain both a functional T-helper determinant and a B-cell epitope(s). In addition, these anti-peptide antisera recognised D15 in the immunoblot assay. Since most peptides contain potent functional T-helper determinant(s) and induce strong IgG antibody responses in mammals, they are candidate immunogens for inclusion in an H. influenzae vaccine preparation. D15 peptide-specific antisera cross-reacted with D15 from non-typeable strains of H. influenzae as judged by immunoblotting. This finding indicates that immunogenic D15 peptides contain epitopes which are highly conserved among typeable and non-typeable strains of H. influenzae. In addition, polyclonal antibodies against these epitopes are useful to detect H. influenzae in biological samples.

Therefore, these conserved epitopes of D15 can be used either individually or in combination to prepare cross-reactive synthetic immunogens against typeable and non-typeable strains of H. influenzae and other bacteria that produce D15 protein, a fragment or an analog thereof. Peptides described above can be further polymerized, or modified with lipids as lipopeptides, or

linked to polysaccharides including PRP as synthetic glycopeptide or lipoglycopeptide conjugates to produce alternate vaccines. These vaccines can be used to immunize against diseases caused by H. influenzae when administered to mammals, for example, by the intramuscular or parenteral route, or when delivered using microparticles, capsules, liposomes and targeting molecules, such as toxins or fragments thereof, and antibodies, to cells of the immune system or mucosal surfaces.

(xiv) Utility of D15 as carrier protein for the production of glycoconjugates.

To determine whether D15 may serve both as a protective antigen and a carrier, D15-PRP conjugation experiments were performed as described in Example 14. The D15-PRP conjugates were found to be highly immunogenic in rabbits and able to elicit both anti-D15 and anti-PRP IgG antibody responses as judged by D15-specific ELISA and PRP-BSA immunoassay (Table 9). These results clearly demonstrate the practical utility of D15 as a carrier protein for glycoconjugation technology.

In preferred embodiments of the present invention, the carrier function of D15 can be generally utilized to prepare chimeric molecules and conjugate vaccines against pathogenic bacteria, including encapsulated bacteria. Thus, the glycoconjugates of the present inventions may be applied to vaccinations to confer protection against infection with any bacteria having polysaccharide antigens, including, for example, Haemophilus influenzae, Streptococcus pneumoniae, Escherichia coli, Neisseria meningitidis, Salmonella typhi, Streptococcus mutans, Cryptococcus neoformans, Klebsiella, Staphylococcus aureus and Pseudomonas aeruginosa.

In another embodiment, the carrier function of D15 may be used, for example, to induce immunity toward abnormal polysaccharides of tumor cells, or to produce

anti-tumor antibodies that can be conjugated to chemotherapeutic or bioactive agents.

Accordingly, the present invention provides the primary sequence and the preparation of an antigen (D15) of H. influenzae that can be used in the prevention and diagnosis of diseases caused by Haemophilus. In particular, the inventors discovered that recombinant D15 or its fragments, can elicit protective antibody responses against live H. influenzae type b bacteria challenge. Thus, the present inventions have utility in vaccines. The invention also discloses the nucleotide sequences of the D15 genes isolated from both H. influenzae type b strains and non-typeable isolates. The DNA segments encoding D15 are disclosed and show minor polymorphism in both their nucleotide and derived amino acid sequences (Figures 1F and 3). These DNA segments may be used to provide an immunogen essentially free from other H. influenzae antigens (such as PRP and lipooligosaccharides (LOS)) through the application of recombinant DNA technology. The present disclosure further provides novel techniques which can be employed for preparing essentially pure D15 or fragments thereof, as well as functional analogs. The recombinant D15 protein, fragment or analog thereof, may be produced in a suitable expression system, such as E. coli, Haemophilus Bordetella, Bacillus, Fungi, Yeast, Baculovirus, Poxvirus, vaccinia or mammalian expression systems.

In one embodiment, the present invention concerns the process of preparing vaccine compositions which include purified recombinant D15 protein (rD15) or rD15 fragments that are immunologically cross-reactive with native D15. In particular, the gene coding the entire D15 protein and a DNA segment encoding an N-terminal rD15 fragment fused to the glutathione-S-transferase gene have been constructed and expressed in E. coli. The expressed

rD15 protein and its fragments were found to cross-react immunologically with the native D15 antigen isolated from both typeable and non-typeable H. influenzae isolates and thus represent cross-reactive immunogens for inclusion in a vaccine against diseases caused by H. influenzae. Furthermore, Haemophilus convalescent serum recognized D15 purified from H. influenzae as described herein, rD15 and N-terminal rD15 fragment.

In another embodiment, the present invention provides a gene coding for the outer membrane protein D15 from H. influenzae having the specific nucleotide sequences described herein or ones substantially homologous thereto (i.e. those which hybridize under stringent conditions to such sequences), for genetically engineering hybrids or chimeric proteins containing a D15 fragment fused to another polypeptide or protein or a polysaccharide, such as H. influenzae outer membrane proteins, for example, P1, P2, or P6 or PRP. As a result, the hybrids, chimeric proteins or glycoconjugates may have higher protectivity against H. influenzae than D15, or P1, or P2, or P6, or PRP alone.

Thus, D15 outer membrane protein can function both as a protective antigen and as a carrier in a conjugate vaccine to provide autologous T-cell priming, wherein the hapten part of the conjugate is the capsular polysaccharide moiety (PRP) of H. influenzae. This D15-carbohydrate conjugate can elicit antibodies against both PRP and D15, and thus should enhance the level of protection against H. influenzae-related diseases, especially in infants.

In another embodiment, the present invention comprises an essentially pure form of at least one protein or peptide containing an amino acid sequence corresponding to at least one antigenic determinant of D15, which peptide is capable of eliciting polyclonal antibodies against H. influenzae in mammals. These D15-

specific antibodies are useful in test kits for detecting the presence of H. influenzae in biological samples. The peptides can have, for example, the amino acid sequences corresponding to residues 20-49, 45-74, 68-99, 93-122, 114-143, 135-164, 157-187, 180-209, 199-228, 219-249, 241-270, 262-291, 282-312, 304-333, 325-354, 346-375, 367-396, 390-416, 410-435, 430-455, 450-477, 471-497, 491-516, 511-538, 532-559, 554-582, 577-602, 596-625, 619-646, 641-666, 662-688, 681-709, 705-731, 725-750, 745-771, 769-798 (SEQ ID NOS: 14 to 49) of the D15 protein of the H. influenzae type b Ca strain, respectively, as set forth in Table 2 below, or any portion, variant or mutant thereof which retains immunogenicity.

In yet another embodiment, the present invention provides pure native D15 protein, extracted and chromatographically purified from cultures of H. influenzae typeable or non-typeable isolates. The novel procedures involves extraction of the D15 protein from cell paste by techniques known for other outer membrane proteins, with an aqueous detergent solution, followed by purification by centrifugation and chromatography. The purified native D15 antigen can be used to immunize mammals against diseases caused by H. influenzae, for example, by the intramuscular or the parenteral routes, or by delivering it using microparticles, capsules, liposomes and targeting molecules, such as toxins or fragments thereof, and antibodies.

Another aspect of the present invention is that the D15 outer membrane protein, fragments or analogs thereof or peptides corresponding to portions of D15 may be components of a multivalent vaccine against otitis media. This multivalent vaccine comprises at least one immunogenic determinant of D15 as described herein, along with at least one protective antigen isolated from Streptococcus pneumoniae, Branhamella (Moroxella)

catarrhalis, Staphylococcus aureus, or respiratory syncytial virus, in the presence or absence of adjuvant.

The D15 peptides (Table 2) or any portion, variant or mutant thereof, can easily be synthesized either manually or with a commercially available peptide synthesizer, such as the Applied Biosystems Model 430A synthesizer.

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of vaccination, diagnosis, and treatment of diseases caused by Haemophilus infections, and the generation of immunological reagents. A further non-limiting discussion of such uses is further presented below.

1. Vaccine preparation and use

Immunogenic compositions, suitable for use as vaccines, may be prepared from immunogenic D15 outer membrane protein, fragments or analogs thereof and/or peptides corresponding to portions of D15 as disclosed herein. The vaccine elicits an immune response which produces antibodies, including anti-D15 outer membrane protein antibodies and antibodies against D15 that are opsonizing or bactericidal. Should the vaccinated subject be challenged by Haemophilus, the antibodies bind to the D15 outer membrane protein and thereby inactivate the bacterium. Opsonizing and bactericidal antibodies represent examples of antibodies useful in protection against disease.

Vaccines containing peptides are generally well known in the art, as exemplified by U.S. Patents 4,601,903; 4,599,231; 4,599,230; and 4,596,792; all of which references are incorporated herein by reference. As to any further reference to patents and references in this description, they are as well hereby incorporated by reference without any further notice to that effect. Vaccines may be prepared as injectables, as liquid

solutions or emulsions. The D15 outer membrane protein, fragments or analogs thereof or peptides corresponding to portions of D15 may be mixed with physiologically-acceptable excipients which are compatible with the D15 outer membrane protein, fragments, analogs or peptides. Excipients may include, water, saline, dextrose, glycerol, ethanol, and combinations thereof. The vaccine may further contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness of the vaccines. Methods of achieving adjuvant effect for the vaccine includes use of agents, such as aluminum hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in phosphate buffered saline. Vaccines may be administered parenterally, by injection subcutaneously or intramuscularly. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkalene glycols or triglycerides. Oral formulations may include normally employed incipients such as, for example, pharmaceutical grades of saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of the D15 outer membrane protein, fragment analogs and/or peptides.

The vaccines are administered in a manner compatible with the dosage formulation, and in an amount which is therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize antibodies, and if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner.

However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms of the D15 outer membrane protein, analog, fragment and/or peptides. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage of the vaccine may also depend on the route of administration and varies according to the size of the host.

The nucleic acid molecules encoding the D15 outer membrane protein of the present invention may also be used directly for immunization by administration of the DNA directly, for example, by injection for genetic immunization or by constructing a live vector, such as Salmonella, BCG, adenovirus, poxvirus or vaccinia. A discussion of some live vectors that have been used to carry heterologous antigens to the immune system are discussed in, for example, O'Hagan (1992). Processes for the direct injection of DNA into test subjects for genetic immunization are described in, for example, Ulman et al. (1993).

The use of peptides in vivo may first require their chemical modification since the peptides themselves may not have a sufficiently long serum and/or tissue half-life. Such chemically modified peptides are referred to herein as peptide analogs. The term peptide analog extends to any functional chemical equivalent of a peptide characterized by its increased stability and/or efficacy in vivo or in vitro in respect of the practice of the invention. The term peptide analog is also used herein to extend to any amino acid derivative of the peptides as described herein. Peptide analogs contemplated herein are produced by procedures that include, but are not limited to, modifications to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide synthesis and the use of

cross-linkers and other methods which impose conformational constraint on the peptides or their analogs.

Examples of side chain modifications contemplated by the present invention include modification of amino groups, such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidation with methylacetimidate; acetylation with acetic anhydride; carbamylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6, trinitrobenzene sulfonic acid (TNBS); alkylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxa-5'-phosphate followed by reduction with $NaBH_4$.

The guanidino group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2, 3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation via o-acylisourea formation followed by subsequent derivatisation, for example, to a corresponding amide.

Sulfhydryl groups may be modified by methods, such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of mixed disulphides with other thiol compounds; reaction with maleimide; maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulfonic acid, phenylmercury chloride, 2-chloromercuric-4-nitrophenol and other mercurials; carbamylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphonyl halides. Tryosine residues may be altered by

nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids.

2. Immunoassays

The D15 outer membrane protein, analog, fragment and/or peptides of the present invention are useful as antigens in immunoassays, including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known to the art for the detection of anti-bacterial, Haemophilus, D15 and/or peptide antibodies. In ELISA assays, the D15 outer membrane protein, fragment or analogs thereof and/or peptides corresponding to portions of D15 outer membrane protein are immobilized onto a selected surface, for example, a surface exhibiting a protein affinity, such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed D15 outer membrane protein, analog, fragment and/or peptides, a nonspecific protein, such as bovine serum albumin (BSA) or casein, that is known to be antigenically neutral with regard to the test sample may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus decreases the background caused by nonspecific bindings of antisera onto the surface. Normally, the peptides

employed herein are in the range of 12 residues and up and preferably 14 to 30 residues.

The immobilizing surface is then contacted with a sample such as clinical or biological materials to be tested in a manner conducive to immune complex (antigen/antibody) formation. This may include diluting the sample with diluents, such as BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from 2 to 4 hours, at temperatures, such as of the order of 25° to 37°C. Following incubation, the sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution such as PBS/Tween, or a borate buffer.

Following formation of specific immunocomplexes between the test sample and the bound D15 outer membrane protein, analog, fragment and/or peptides, and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined by subjecting the immunocomplex to a second antibody having specificity for the first antibody. If the test sample is of human origin, the second antibody is an antibody having specificity for human immunoglobulins and, in general, IgG. To provide detecting means, the second antibody may have an associated activity, such as an enzymatic activity that will generate, for example, a color development upon incubating with an appropriate chromogenic substrate. Quantification may then achieved by measuring the degree of color generation using, for example, a visible spectra spectrophotometer.

3. Use of sequences as hybridization probes

The nucleotide sequences of the present invention, comprising the sequence of the D15 outer membrane protein, now allow for the identification and cloning of the D15 outer membrane protein genes from any species of

Haemophilus and other bacteria that have genes encoding D15 outer membrane proteins.

The nucleotide sequences comprising the sequence encoding the D15 outer membrane protein of the present invention are useful for their ability to selectively form duplex molecules with complementary stretches of other D15 genes. Depending on the application, a variety of hybridization conditions may be employed to achieve varying degrees of selectivity of the probe toward the other D15 genes. For a high degree of selectivity, stringent conditions are used to form the duplexes, such as low salt and/or high temperature conditions, such as provided by 0.02 M to 0.15 M NaCl at temperatures of between about 50°C to 70°C. For some applications, less stringent hybridization conditions are required such as 0.15 M to 0.9 M salt, at temperatures ranging from between about 20°C to 55°C. Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex. Thus, particular hybridization conditions can be readily manipulated, and will generally be a method of choice depending on the desired results.

In a clinical diagnostic embodiment, the nucleic acid sequences of the D15 outer membrane protein genes of the present invention may be used in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of providing a detectable signal. In some diagnostic embodiments, an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of a radioactive tag may be used. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide means visible to the human eye or

spectrophotometrically, to identify specific hybridization with samples containing D15 gene sequences.

The nucleic acid sequences of D15 genes of the present invention are useful as hybridization probes in solution hybridizations and in embodiments employing solid-phase procedures. In embodiments involving solid phase procedures, the test DNA (or RNA) from samples, such as clinical samples, including exudates, body fluids (e. g., serum, amniotic fluid, middle ear effusion, sputum, bronchoalveolar lavage fluid) or even tissues, is adsorbed or otherwise affixed to a selected matrix or surface. The fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes comprising the nucleic acid sequences of the D15 genes or fragments thereof of the present invention under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required depending on, for example, on the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe etc. Following washing of the hybridization surface so as to remove non-specifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label. The selected probe should be at least 18 bp and may be in the range of 30 bp to 90 bp long.

4. Expression of the D15 outer membrane protein genes

Plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell may be used for the expression of the D15 outer membrane protein genes in expression systems. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, E. coli may be transformed using pBR322 which contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells.

The pBR322 plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins.

In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as a transforming vector in connection with these hosts. For example, the phage in lambda GEM™-11 may be utilized in making recombinant phage vectors which can be used to transform host cells, such as E. coli LE392.

Promoters commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems and other microbial promoters, such as the T7 promoter system. Details concerning the nucleotide sequences of promoters are known, enabling a skilled worker to ligate them functionally with plasmid vectors. The particular promoter used generally is a matter of choice depending upon the desired results. Hosts that are appropriate for expression of the transferrin receptor genes, fragment analogs or variants thereof include E. coli, Bacillus, Haemophilus, Bordetella, fungi, yeast, or the baculovirus and poxvirus expression systems may be used.

In accordance with an aspect of this invention, it is preferred to make the D15 outer membrane protein, fragment or analog thereof by recombinant methods, particularly since the naturally occurring D15 protein as purified from culture of a species of Haemophilus may include undesired contaminants, including trace amounts of toxic materials. This problem can be avoided by using recombinantly produced D15 outer membrane protein in heterologous systems which can be isolated from the host in a manner to minimize toxins in the purified material. Particularly desirable hosts for expression in this regard include Gram positive bacteria which do not have

lipopolysaccharide (LPS) and are, therefore, endotoxin free. Such hosts include species of Bacillus and may be particularly useful for the production of non-pyrogenic D15 outer membrane protein, fragments or analogs thereof.

BIOLOGICAL DEPOSITS

Certain plasmids that contain at least a portion coding for a D15 outer membrane protein from strains of Haemophilus influenzae that are described and referred to herein have been deposited with the American Type Culture Collection (ATCC) located at Rockville, Maryland USA pursuant to the Budapest Treaty and prior to the filing of this application. Samples of the deposited plasmids will become available to the public upon grant of a patent based upon this United States patent application. The invention described and claimed herein is not to be limited in scope by plasmids deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent or similar plasmids that encode similar or equivalent antigens as described in this application are within the scope of the invention.

DEPOSITE SUMMARY

Clone	H. influenzae	ATCC Designation	Date Deposited
DS-712-2-1	Eagan	75604	November 4, 1993
JB-1042-5-1	SB33	75606	November 4, 1993
DS-880-1-2	Eagan	75605	November 4, 1993

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of

the invention. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations. Immunological and recombinant DNA methods may not be explicitly described in this disclosure but are well within the scope of those skilled in the art.

EXAMPLES

Methods of molecular genetics, protein biochemistry, and immunology used but not explicitly described in this disclosure and these EXAMPLES are amply reported in the scientific literature and are well within the ability of those skilled in the art.

Example 1

This Example illustrates the cloning and sequencing of the D15 genes.

Genomic DNA was purified from the Haemophilus influenzae type b strain Ca by lysis of the bacteria with pronase and sodium dodecylsulphate followed by phenol extraction and isopropanol precipitation, according to Berns and Thomas, 1965. The DNA was then partially digested with EcoRI and the DNA fraction containing 6-10 kb fragments was isolated following electrophoresis in low-melting point agarose. These fragments were ligated into a lambda gt11 Amp1 vector (Thomas and Rossi, 1986) and cloned as a lysogen into E. coli strain BTA282. Recombinant clones were selected for their ampicillin resistance conferred by the vector. To identify clones producing H. influenzae type b antigen, the clones were replica-plated on nitrocellulose filters and duplicate colonies induced for expression by temperature switch to 42°C for 2 hours. Colonies were lysed by wetting the filters with 1% sodium dodecylsulphate (SDS). The filters were then placed into a chloroform-saturated atmosphere for 15 min. The filters were then assayed by colony radioimmuno-assay using a hyperimmune rabbit anti-H. influenzae type b antiserum absorbed with E. coli lysate

for antigen expression. Clones shown by autoradiography to be producing H. influenzae type b antigens were further purified and their replicates retested for reactivity with the hyperimmune anti-H. influenzae type b antiserum. The antiserum absorbed with 10^{10} intact H. influenzae type b bacteria (strain Ca) was used as negative control.

A number of clones were identified which reacted with the unabsorbed, but not with the absorbed antiserum and were further analysed. One of the clones, D15, was purified, grown and found to produce a H. influenzae type b antigen which migrated in sodium dodecyl sulphate polyacrylamide gels with a M_r of about 80 kDa. Lysates from the D15 clone were coupled to Sepharose™ 4B gel and used to affinity-purify anti-D15 antibodies. This procedure is described by Thomas et al, 1990, except that the apparent M_r was initially reported to be about 103 kDa. The affinity-purified antibodies to D15 were then shown to react with an M_r 80 kDa protein in an outer membrane protein preparation of H. influenzae type b (sarcosyl insoluble fraction - Carlone et al, 1986). Radioimmuno dot blots and Western blots analyses of membrane preparations from both type b and nontypeable Haemophilus influenzae strains showed that affinity-purified anti-D15 antibodies reacted with all isolates. These antibodies were found to be capable of passively protecting infant rats from bacteraemia following intraperitoneal injection of live H. influenzae type b bacteria. The specificity of the protection was confirmed by absorbing out the protective activity of anti-D15 antibodies with a lysate of E. coli expressing D15 coupled to Sepharose. The protection studies have been described in detail by Thomas et al, 1990.

DNA from the lambda gt11 Amp1 D15 phage was isolated and a 5.7 kb fragment was released by EcoRI digestion. This fragment was subcloned into pUC19 and the resulting

plasmid transformed into E. coli HB101. Recombinant bacteria were found to produce the expected M_r 80 kDa H. influenzae type b antigen when examined by Western blotting. The insert DNA was then characterised by restriction endonuclease mapping. A 2.8 kb HindIII-EcoRI fragment was subcloned into pUC19 to generate plasmid pUC19/D15, which was transformed into E. coli HB101. The recombinant bacteria expressed a M_r 80 kD protein recognized by D15-specific antibodies on Western blot analysis of E. coli lysates.

Plasmid DNA was prepared from two individual colonies of recombinant E. coli HB101 containing the pUC19/D15 plasmid using standard techniques. Oligonucleotide sequencing primers of 17-25 bases in length were synthesized on the ABI model 380B DNA Synthesizer and purified by chromatography using OPC cartridges obtained from Applied Biosystems Inc., and used in accordance with the manufactures recommendations. Samples were sequenced using the ABI model 370A DNA Sequencer and dye terminator chemistry according to manufacturers' protocols. This sequence analysis indicated that the D15 gene contains an open reading frame encoding for 789 amino acids, including a putative signal sequence (Figure 1). The derived amino acid sequence was found to contain the sequence of an internal peptide obtained by thrombin digestion of native D15 that had been chemically determined. The amino acid composition of D15 derived from the D15 gene sequence was comparable (within experimental error) to that of the native protein as determined by amino acid analysis.

Example 2

This Example illustrates the preparation of chromosomal DNA from Haemophilus influenzae strains Eagan, MinnA, SB33, and PAK 12085.

H. influenzae strains were grown on Mueller-Hinton agar or in brain heart infusion broth as described by Harkness et al., 1992.

Eagan chromosomal DNA

Bacteria from 50 mL of culture were pelleted by centrifugation at 5,000 rpm, 20 minutes, 4°C. The pellet was resuspended in 25 mL TE (10mM Tris, 1mM EDTA, pH 8.0) and 2 x 5mL aliquots used for chromosomal DNA preparation. To each aliquot were added 0.6 mL of 10% sarkosyl and 0.15 mL of 20mg/mL proteinase K and the samples incubated at 37°C for 1 hour. The lysate was extracted once with Tris-saturated phenol (pH 8.0) and three times with chloroform:isoamyl alcohol (24:1). The aqueous phase was pooled for a final volume of 7 mL. Then, 0.7 mL of 3M sodium acetate (pH 5.2) and 4.3 mL of isopropanol were added to precipitate the DNA which was spooled, rinsed with 70% ethanol, dried, and resuspended in 1 mL of water.

MinnA, SB33, and PAK 12085 chromosomal DNA

Bacteria from 50 mL of culture were pelleted by centrifugation at 5,000 rpm for 15-20 minutes, at 4°C, in a Sorvall RC-3B centrifuge. The cell pellet was resuspended in 10 mL of TE (10mM Tris-HCl, 1mM EDTA, pH 7.5), pronase was added to 500 μg/mL, and SDS to 1%. The sample was incubated at 37°C for about 4 hours until a clear lysate was obtained. The lysate was extracted once with Tris-saturated phenol, once with Tris-saturated phenol/chloroform (1:1), and once with chloroform. The final aqueous phase was dialysed for 24 hours against 2 x 500 mL of 1M NaCl at 4°C, changing the buffer once, and for 24 hours against 2 x 500 mL of TE at 4°C, changing the buffer once. The final dialysate was aliquotted for subsequent use.

Example 3

This Example illustrates the preparation of Haemophilus influenzae chromosomal libraries.

H. influenzae Eagan and PAK 12085 chromosomal DNAs were digested with Sau3A I (0.5 unit/10 μg DNA) at 37°C for 15 minutes and size-fractionated by agarose gel electrophoresis. Gel slices corresponding to DNA fragments of 15-23 kb were excised and DNA was electroeluted overnight in dialysis tubing containing 3 mL of TAE (40mM Tris-acetate, 1mM EDTA, pH 8.0) at 14V. The DNA was precipitated twice and resuspended in water before overnight ligation with EMBL3 BamH I arms (Promega). The ligation mixture was packaged using the Lambda in vitro packaging kit (Amersham) according to the manufacturer's instructions and plated onto E. coli NM539 cells. The library was titrated, then amplified and stored at 4°C under 0.3% chloroform.

MinnA chromosomal DNA (10 μg) was digested with Sau3A I (40 units) for 2, 4, and 6 minutes then size-fractionated on a 10-30% sucrose gradient in TNE (20mM Tris-HCl, 5mM NaCl, 1mM EDTA, pH 8.0). Fractions containing DNA fragments >5 kb were pooled and precipitated. In a second experiment, chromosomal DNA (2.6 μg) was digested with Sau3A I (4 units) for 1, 2, and 3 minutes and size- fractionated by preparative agarose gel electrophoresis. Gel slices containing DNA fragments of 10-20 kb were excised and DNA extracted by a standard freeze/thaw technique. The size-fractionated DNA from the two experiments was pooled for ligation with BamH I arms of EMBL3 (Promega). The ligation mix was packaged using the Gigapack II packaging kit (Amersham) and plated on E. coli LE392 cells. The library was titrated, then amplified and stored at 4°C under 0.3% chloroform.

SB33 chromosomal DNA (20 μg) was digested with Sau3A I (40 units) for 2, 4, or 6 minutes and size-fractionated on a 10-30% sucrose gradient in TNE (20mM Tris-HCl, 5mM NaCl, 1mM EDTA, pH 8.0). Fractions containing fragments >5 kb were pooled. In a second experiment, SB33

chromosomal DNA (2 μg) was digested with Sau3A I (4 units) for 2, 4, or 6 minutes and size-fractionated on a preparative agarose gel. Gel slices containing DNA fragments of 10-20 kb were excised and DNA extracted by a standard freeze/thaw technique. The size-fractionated DNA from both experiments was pooled for ligation with BamH I arms of EMBL3 (Promega). The ligation mix was packaged using the Gigapack II packaging kit and plated on LE392 cells. The library was titrated, then amplified and stored at 4°C under 0.3% chloroform.

Example 4

This Example illustrates the screening of the DNA libraries.

The Eagan, MinnA, SB33, and PAK 12085 DNA libraries were plated onto LE392 cells on NZCYM plates using 0.7% top agarose in NZCYM as overlay. Plaque lifts onto nitrocellulose filters were performed following standard procedures, and filters were processed and hybridized with a digoxigenin-labelled D15 probe prepared according to the manufacturer's specifications (Boehringer Mannheim). The probe was the EcoR I/Hind III fragment from pUC19/D15 containing the entire Ca D15 gene (Figure 2). Putative plaques were plated and submitted to a second round of screening using the same procedures. Phage DNA was prepared from 500 mL of culture using standard techniques, the insert DNA was excised by Sal I digestion, and cloned into pUC to generate clones DS-712-2-1 (Eagan), DS-691-1-5 (MinnA), JB-1042-5-1 (SB33), and JB-1042-9-4 (PAK 12085), which are shown in Figure 2.

The nucleotide sequences of the D15 genes from H. influenzae type b strains Eagan and MinnA the non-typeable H. influenzae strains SB33 and PAK 12085 were determined and compared with that for strain Ca, as seen in figures 1b, 1C, 1D, 1E and 1F. The desired amino acid sequence are shown in Figures 1B, 1C, 1D and 1E and are

compared with the amino acid sequence of the D15 protein of H. influenzae type b Ca (Figure 3).

Example 5

This Example illustrates the expression of rD15 protein in E. coli.

A 2.8 kb fragment HindIII-EcoRI was subcloned into pUC19 and this pUC19/D15 plasmid was transformed into E. coli HB101. Upon induction, the positive clones expressed an 80 kDa protein which was recognized by D15-specific antisera on Western blot analysis. A HindIII-Pst I fragment was also subcloned into pUC19 and shown to express a 67 kDa protein. According to the restriction map, this 67 kDa protein corresponded to a C-terminal truncated D15 protein. On Western blot analysis, this truncated D15 was still recognized by the D15-specific antisera.

Plasmids to express the D15 gene of the non-typeable strain SB33 in E. coli were constructed. Plasmid JB-1042-5-1 containing the SB33 D15 gene and its flanking regions, was digested with EcoR I and Hind III and the 3kb D15 insert subcloned into pUC to give plasmid pRY-60-1 (Figure 4). Appropriate oligonucleotides were synthesized to restore the native D15 sequence between the ATG codon of the expression plasmid pT7-7 and the BsrF I site within the D15 gene. These oligonucleotides had the following sequence:

Nde

5'- TATGGCACCTTTTGTGGCAAAAGATATTCGTGTGGATGGTGTTCAAGGTG

ACCGTGGAAAACACCGTTTTCTATAAGCACACCTACCACAAGTTCCACTGAATCT

ACTTAGAATCAACAAACCGAGCAAGTTTACCTGTTCGTG - SEQ ID NO: 50

TGGTTGTTTAGGCTCGTTCAAATGGACAAGCACGGCC-5'- SEQ ID NO: 51

BsrF I

Plasmid pRY-60-1 was digested with EcoR I and BsrF I and the DNA fragment containing most of the D15 gene was purified. pUC was digested with EcoR I and Nde I and the

vector fragment purified. A multi-component ligation between the pUC and D15 fragments and the oligonucleotides generated plasmid DS-860-1-1 which contains a D15 sequence without a promoter. pT7-7 was digested with Nde I and EcoR I and the vector fragment purified. DS-860-1-1 was digested with Nde I and EcoR I and the D15 insert was purified and ligated with the T7-7 vector generating plasmid DS-880-1-2 (Figure 4).

The plasmid constructions were performed using E. coli JM109 as host. For expression, plasmid DS-880-1-2 was transformed into E. coli BL21/DE3, BL21/DE3/pLysS, or JM109/DE3 cells. Transformation of the cells was performed using either calcium chloride-treated competent cells or by electroporation using a BioRad electroporator. Transformed cells were grown in YT, M9, or NZCYM media and induced with IPTG or other inducing agents.

Example 6

This Example illustrates the construction and expression of the GST-D15 fragment hybrid gene in E. coli.

A forward sense primer (primer 1) 5'-GGGGAATTCCAAAAGATGTTCGT (SEQ ID NO: 52) and a reverse antisense primer CACGAATTCCCTGCAAATC-5' (primer 7 - SEQ ID NO: 53) were used to amplify a 2.8 Kb fragment HindIII-EcoRI of the D15 gene by the polymerase chain reaction that encodes the N-terminal amino acid residues 22 to 223 of the primary sequence of D15 protein (Figure 1A). The nucleotide sequence of the 609bp amplified fragment was confirmed by DNA sequencing. The amplified gene segment was ligated into the pGEX-2T vector downstream from the GST gene and transformed into E. coli TG-1. Colonies expressing the H. influenzae type b antigen were screened with a rabbit anti-H. influenzae type b antiserum by colony radioimmunoassay and isolated. The glutathione-S-transferase-D15 fragment fusion protein

produced by transformed E. coli was isolated by affinity purification on glutathione agarose.

Example 7

This Example describes alternative expression systems for rD15.

The D15 gene or fragments thereof are also expressed in E. coli under the control of other regulated promoters. The D15 gene or fragments thereof are expressed in the absence of the leader peptide, or in other cloning systems where toxicity of D15 expression to the host is not problematic. The gene or fragments thereof are synthesized de novo or by employing the polymerase chain reaction using suitable primers. These genes are cloned into suitable cloning vectors or bacteriophage vectors in E. coli or other suitable hosts directly when toxicity can be avoided. Expression systems are Gram-positive bacteria (such as Bacillus species), pox virus, adenovirus, baculovirus, yeast, fungi, BCG or mammalian expression systems.

Example 8

This Example illustrates the protocol for extraction and purification of rD15 from E. coli expression system.

The cell pellet from a 250 mL culture, prepared as described in Example 5, was resuspended in 40 mL of 50 mM Tris, pH 8.0, and disrupted by sonication (3 x 10 min, 70% duty circle). The extract was centrifuged at 20,000 x *g* and the resulting pellet saved. The initial pellet was re-extracted with 40 mL of 50 mM Tris, 0.5% Triton X-100, 10 mM EDTA, pH 8.0. The suspension was then sonicated for 10 minutes at 70% duty circle. The extract was centrifuged at 300 x *g* for 5 minutes. The resulting supernatant was centrifuged again at 20,000 x *g* for 30 min and the resulting pellet was saved. The pellet was resuspended in 50 mM Tris, 0.5% Triton X-100, 10 mM EDTA, pH 8.0. The suspension was then mixed with PBS/ 8 M urea to a final urea concentration of 6 M. The solution was

then dialyzed against PBS to remove urea. After dialysis, the solution was centrifuged at 300 x *g* for 10 min., the supernatant was saved and stored at 4°C.

Example 9

This Example demonstrates the purification of GST-(D15 fragment) fusion protein using glutathione-Sepharose 4B affinity chromatography.

Five mg of GST-(D15 fragment) fusion protein crude extract, prepared as described in Example 6, were dissolved in 5 mL of phosphate buffer saline (PBS) containing 1% Triton X-100. The solution was then loaded onto a Glutathione-Sepharose 4B column (2 mL) equilibrated with PBS containing 1% Triton X-100. The run-through of the column was discarded. The column was washed with 20 mL of PBS and the GST-(D15 fragment) fusion protein was eluted with 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM glutathione. Elution was monitored by absorbance at 280 nm. Protein-containing fractions (2 mL/fraction) were collected and pooled. The purity of the protein was assessed by SDS-PAGE (Figure 9, lane 3). The final volume of the purified fusion protein was 6 mL.

Example 10

This Example illustrates the protocol used for thrombin digestion of proteins to release the truncated D15 molecule.

The GST-(D15 fragment) fusion protein sample from Example 9 (0.1 to 0.5 mg protein/mL) was dialyzed against 1 L of 50 mM Tris-HCl buffer (pH 8.5) 3 times with at least 2 hour intervals at 4°C to remove protease inhibitors. After dialysis, the solution was treated with human thrombin (Sigma) at a ratio of 1 mL of solution to 25 units of thrombin. The cleavage reaction was carried out at 37°C for 2 hr and analysed by SDS-PAGE (Figure 9, lane 4). The reaction was stopped by placing the solution in ice.

Example 11

This Example illustrates the procedure used for N-terminal rD15 fragment purification from GST using Glutathione-Sepharose 4B affinity chromatography.

A thrombin-digested GST-(D15 fragment) sample, prepared as described in Example 10, was loaded onto a Glutathione-Sepharose 4B column (2 mL) equilibrated with PBS containing 1% Triton X-100. The run-through of the column containing the N-terminal rD15 fragment was saved. After washing the column with 20 mL of PBS, the affinity column was regenerated by removing GST using 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM glutathione. The purity of rD15 fragment was analysed by SDS-PAGE (Figure 9, lane 5). This N-terminal rD15 fragment contains amino acids 63-223 of the D15 protein as a result of cleavage at the spacious thrombin site shown in Figure 1A.

Example 12

This Example illustrates the protocol used for the purification of D15-specific polyclonal antibodies by affinity chromatography using GST-(D15 fragment) fusion protein.

The recombinant GST-(D15 fragment) fusion protein, prepared as described in Example 9, was conjugated to cyanogen bromide-activated Sepharose. The affinity column was then used to purify antibodies from a rabbit hyperimmune anti-H. influenzae type b antiserum. The affinity purified-antibodies were shown by immunoblotting to react with a 80 kDa component present in the lysates of E. coli transformed with pUC9/D15 and in the lysates of several typeable and nontypeable H. influenzae isolates. These results confirmed that the DNA segment encoding the D15 fragment of the fusion protein was part of the open reading frame of the D15 gene.

Similarly, antisera raised against the recombinant fusion protein (Example 9) or the purified N-terminal rD15 fragment (Example 11) reacted with the D15 protein produced by H. influenzae strains (Example 13).

Example 13

This Example describes the protocol used for the purification of native D15 from H. influenzae.

Cell paste of the non-typeable H. influenzae SB33 strain, prepared from a culture grown in brain heart infusion medium supplemented with NAD (2μg/mL) and HEMIN (2μg/mL) at 37°C, as described in Panezutti, et al, 1993, was resuspended in 50 mM Tris-HCl, pH 8.0, containing 0.5% Triton X-100 and 10 mM EDTA (20 mL per 1 g of cell paste). The mixture was stirred at room temperature for 2 hr, then centrifuged at 20,000 x *g* for 30 minutes. The D15 was located in the supernatant and further purified.

Purification of native D15 was achieved by affinity chromatography using a D15-specific monoclonal antibody (see Example 24). The D15 extract (25 mL) was mixed with the affinity matrix (1 mL) at room temperature for 2 hr. The mixture was packed into a column and the run-through fraction was discarded. The column was washed sequentially with the following buffers: 50 mM Tris-HCl, pH 8.0, containing 0.5% Triton X-100 and 10 mM EDTA; 1 M HEPES buffer, pH 6.8; 50 mM Tris-HCl, pH 8.0, containing 0.5% Triton X-100 and 10 mM EDTA; and 10 mM phosphate buffer, pH 8.0. D15 was then eluted from the column with 3 mL of 50 mM diethylamine, pH 12.0 and the protein solution was neutralized by 1 M HEPES, pH 6.8 (1/10 volume). The affinity-purified native D15 was analysed by SDS-PAGE and stored at -20°C.

Example 14

This Example describes the procedure used for the preparation of D15-PRP conjugates.

Haemophilus influenzae type b oligosaccharides (PRP) prepared by controlled acid hydrolysis were conjugated either with the purified native (Example 13) or recombinant D15 (Example 8) as well as with its fragments (Example 11) using periodate oxidation as described in US Patent 4356170 and further details of which are presented

in Example 17. The mean molecular size of the PRP molecules used for conjugation was determined as being approximately 20,000 Daltons. The conjugation was carried out without a linker molecule but may also be carried out with a linker molecule. A PRP/D15 molar ratio of approximately 7 was used to provide an excess of PRP hapten.

The PRP/rD15 conjugate was tested according to the protocol of Example 18 for immunogenicity in rabbits and elicited both primary and secondary anti-PRP IgG and anti-D15 antibody responses (Table 9). Rabbit anti-rD15-PRP antisera also strongly reacted with both native D15 and rD15 as judged by immunoblot analysis. These data indicate that rD15 can be used as a carrier protein in a conjugate vaccine. In addition, a rD15-PRP conjugate vaccine should ensure a more consistent protection against H. influenzae type b disease, particularly in infants, as a result of the additional homotypic protection provided by antibodies directed against the D15 protein.

Example 15

This Example describes the preparation of D15 peptides.

D15 peptides (Table 2) were synthesized using an ABI 430A peptide synthesizer and optimized t-Boc chemistry as described by the manufacturer, then cleaved from the resin by hydrofluoric acid (HF). The peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) on a Vydac C4 semi-preparative column (1 x 30 cm) using a 15 to 55% acetonitrile gradient in 0.1% trifluoryl acetic acid (TFA) developed over 40 minutes at a flow rate of 2 mL/min. All synthetic peptides (Table 2) used in biochemical and immunological studies were >95% pure as judged by analytical HPLC. Amino acid composition analyses of

these peptides performed on a Waters Pico-Tag system were in good agreement with their theoretical compositions.

Example 16

This Example describes the protocol used for D15 peptide-specific antisera production.

Guinea pigs and rabbits were immunized with individual peptides (50 to 200 μg) emulsified with Freund's complete adjuvant and injected intramuscularly. After two booster doses with the same amount of peptide in incomplete Freund's adjuvant at +14 and +28 days, the anti-peptide antisera were collected on day +42 and tested by ELISAs and immunoblotting. Both rabbit and guinea pig antisera were shown to be monospecific for their respective immunizing peptides by the peptide-specific ELISAs (Table 6). In addition, both guinea pig and rabbit antisera raised against D15 peptides reacted with both H. influenzae type b and non-typeable D15 on immunoblot analyses. Since most D15 peptides induced strong anti-peptide antibody responses in at least one animal species, they are appropriate immunogens to be included in immunogenic compositions including vaccine preparations.

Example 17

This Example describes the procedure used for the preparation of PRP-BSA conjugates.

0.5 mL of periodate-oxidized PRP (25mg in 1 mL of 0.1 M sodium phosphate buffer, pH 6.0), prepared from native PRP treated with aqueous periodic acid (Carlone et al, 1986), was added to bovine serum albumin (BSA) (1.32 mg ; 0.02 μmol) in 0.5 mL of 0.2 M sodium phosphate buffer, pH 8.0, followed by the addition of sodium cyanoborohydride (14 μg ; 0.22 μmol ; 10 eqv. to BSA). After incubation at 37°C for 5 days, the reaction mixture was dialysed against 4 L of 0.1 M phosphate buffer, pH 7.5. The resulting solution was applied onto an analytical Superose 12 column (15 x 300 mm, Pharmacia)

equilibrated with 0.2 M sodium phosphate buffer, pH 7.2, and eluted with the same buffer. Fractions were monitored for absorbance at 230 nm. The first major protein peak was pooled and concentrated in a Centriprep 30 to 2.2 mL. The amount of protein was determined using the Bio Rad protein assay, and was found to be 300 μg/mL. The presence of PRP in the protein conjugate fraction was confirmed by the Orcinol test.

Example 18

This Example describes the protocol used for the production of anti-PRP antisera in animals using rD15-PRP conjugates.

Rabbits were immunized intramuscularly with rD15-PRP conjugates (Example 14) (5 to 50 μg PRP equivalent) mixed with 3 mg $AlPO_4$ per mL, followed by two booster doses (half amount of the same immunogen) at 2 week intervals. Antisera were collected every 2 weeks after the first injection, heat-inactivated at 56°C for 30 minutes and stored at -20°C.

Example 19

This Example illustrates the reactivity between D15 peptides and anti-peptide and D15-specific antisera using D15-specific and peptide-specific ELISAs.

Microtiter wells (Nunc-Immunoplate, Nunc, Denmark) were coated with 200 ng of purified rD15 or 500 ng of individual peptides in 50 μL of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) for 16 hours at room temperature. The plates were then blocked with 0.1% (w/v) BSA in phosphate buffer saline (PBS) for 30 minutes at room temperature. Serially diluted antisera were added to the wells and incubated for 1 hour at room temperature. After removal of the antisera, the plates were washed five times with PBS containing 0.1% (w/v) Tween-20 and 0.1% (w/v) BSA. $F(ab')_2$ fragments from goat anti-rabbit, guinea pig, mouse, or human IgG antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs Inc.,

PA) were diluted (1/8,000) with washing buffer, and added onto the microtiter plates. After 1 hr incubation at room temperature, the plates were washed five times with the washing buffer. The plates were then developed using the substrate tetramethylbenzidine (TMB) in H_2O_2 (ADI, Toronto). The reaction was stopped with 1N H_2SO_4 and the optical density was measured at 450 nm using a Titretek Multiskan II (Flow Labs., Virginia). Two irrelevant peptides as negative controls in the peptide-specific ELISAs. Assays were performed in triplicate, and the reactive titer of each antiserum was defined as the dilution consistently showing 2-fold increase absorbance value over those obtained from the negative controls. The results obtained are summarized in Tables 3, 6 and 8 and in the DETAILED DESCRIPTION OF THE INVENTION above.

Example 20

This Example illustrates the measurement of the anti-PRP IgG titers in rabbit anti-PRP-D15 conjugate antisera using a PRP-specific ELISA.

Microtiter wells (Nunc-Immunoplate, Nunc, Denmark) were coated with 200 ng of purified PRP-BSA (see Example 17) in 200 μL of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) for 16 hours at room temperature. The plates were then blocked with 0.1% (w/v) BSA in phosphate buffer saline (PBS) for 30 minutes at room temperature. Serially diluted rabbit antisera raised against PRP-D15 conjugates were added to the wells and incubated for 1 hour at room temperature. After removal of the antisera, the plates were washed five times with PBS containing 0.1% (w/v) Tween-20 and 0.1% (w/v) BSA. $F(ab')_2$ fragment from goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs Inc., PA) were diluted (1/8,000) with washing buffer, and added onto the microtiter plates. After 1 hour incubation at room temperature, the plates were washed five times with the washing buffer. The plates were then developed using

the substrate tetramethylbenzidine (TMB) in H_2O_2 (ADI, Toronto). The reaction was stopped with 1N H_2SO_4 and the optical density measured at 450 nm using a Titretek Multiskan II (Flow Labs., Virginia). A standard anti-PRP antiserum of known titer was included as positive control. Assays were performed in triplicate, and the reactive titer of each antiserum was defined as the reciprocal of the dilution consistently showing a 2-fold increase in O.D. value over that obtained with the pre-immune serum (Table 9).

Example 21

This Example describes the protocol used for the production of D15-specific antisera using purified D15, rD15 or N-terminal rD15 fragment.

New Zealand White rabbits (Maple Lane) and guinea pigs (Charles River) were immunized intramuscularly (IM) with a 10 μg dose of either affinity-purified native D15 (Example 13), recombinant D15 (Example 8) or N-terminal rD15 fragment (Example 11) emulsified in Freund's complete adjuvant (Difco). Animals were boosted on day 28 with another 10 μg dose of affinity-purified D15 or rD15 or rD15 fragment emulsified in Freund's incomplete adjuvant and bled on day 42 via the marginal ear vein. D15-specific polyclonal antibodies were purified from this material as described in Example 12.

Example 22

This Example illustrates the protective activity of D15-specific antisera against H. influenzae type b challenge using the infant rat model of bacteremia.

Five-day old infant rats were inoculated subcutaneously (SC) on the dorsum with 0.15 mL of two different rabbit anti-N-terminal rD15 fragments. Pre-immune sera were used as negative controls. One day after immunization, the infant rats were injected intraperitoneally (IP) with 200 colony-forming units (cfu) of Haemophilus influenzae type *b* Minn A strain (0.1

ml) freshly grown in brain heart infusion (BHI) medium supplemented with cofactors and diluted in PBS containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$. One day later, blood samples were collected via cardiac puncture under methoxyflurane anaesthesia and plated on chocolate agar plates. The number of bacteria per mL of blood was quantified after 24 hr. The statistical significance of differences observed in the levels of bacteremia relative to controls was analyzed by the Student's t-test. The results are summarized in Table 1.

Example 23

This Example describes the protocol used for the generation of D15-specific T-cell lines.

BALB/c ($H\text{-}2^d$) mice purchased from Charles River Animal Farm (Montreal, Canada) were individually primed subcutaneously with 20 μg of rD15 adsorbed to 1.5 mg of aluminium phosphate (alum). The animals were boosted twice with the same dose of immunogen at 3 week intervals. Ten days after the final boost, spleens of immunized mice were removed. Splenocytes were cultured at 5.75×10^5 cells per well in a final volume of 200 μL of RPMI 1640 medium (Flow Lab.) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2 mM L-glutamine (Flow Lab.), 100 U/mL) penicillin (Flow Lab.) and 5×10^{-5} M 2-mercaptoethanol (Sigma) in the presence of varying concentrations (1, 10 and 100 μg per mL) of individual D15 peptides (Table 2) in 96-well plates (Nunc, Denmark). Cultures were kept in a humidified incubator in the presence of 5% $C0_2$/air. Triplicate cultures were performed for each concentration of each peptide. Five days later, 150 μL of 10% rat concanavalin A culture supernatant diluted in culture medium was added to the microtiter plate wells as a source of Interleukin-2 (IL-2) to expand peptide-specific T-cells. Six days later, 150 μL of supernatant were removed from each microculture, and 150 μL of fresh IL-2 containing culture

supernatant added to further expand and maintain the viability of the peptide-specific T-cells. After a further 6 day-incubation, the cells were washed three times, each time with 200 μL of culture medium.

Each set of cultures was then stimulated with the corresponding concentrations (1, 10 and 100 μg per mL) of the peptide in the presence of 2 x 10^5 irradiated (1,500 rad) BALB/c spleen cells in a final volume of 200 μL of culture medium. Sixty μL of supernatant were then removed from each microculture. The supernatants from each triplicate cultures set were pooled. All supernatants were assayed for IL-2, Interleukin-4 and Interferon-gamma (IFN-γ). Detections of IL-2 and IL-4 were performed using murine IL-2 and IL-4 ELISA kits purchased from Endogen Inc. (MA, USA) respectively. Assay of IFN-γ was performed using a mouse IFN-γ ELISA kit supplied by Genzyme Corporation (MA, USA). Test culture supernatants were assayed at 1 in 5 dilution according to the manufacturers' instructions. The results obtained are set forth in Table 7.

Example 25

This Example describes the general procedure used for the production of murine D15-specific monoclonal antibodies.

BALB/c mice were immunized intraperitoneally with 20 to 50 μg of the N-terminal rD15 fragment (Example 11) emulsified in Freund's complete adjuvant. Two weeks later, the mice were given another injection of the same amount of immunogen in incomplete Freund's adjuvant (IFA). Three days before the fusion, the mice were boosted again with the same amount of immunogen in IFA. Hybridomas were produced by fusion of splenic lymphocytes from immunized mice with non-secreting Sp2/0 myeloma cells as previously described by Hamel et al. (1987). D15-specific hybridomas were cloned by sequential limiting dilutions and screened for anti-D15 monoclonal

antibody production. Eight D15-specific hybridoma cell lines were identified, expanded and frozen in liquid nitrogen. One of the hybridoma cell lines, 6C8-F6-C6, has been partially characterized. The monoclonal antibody (MAb 6C8-F6-C6) reacts with peptide D15-P8. This MAb 6C8-F6-C6 was used to prepare the D15-specific MAb affinity column and purify native D15 from H. influenzae cell paste (Example 13).

TABLE 1

PROTECTIVE EFFECT OF PASSIVELY TRANSFERRED ANTI-N-TERMINAL RD15 FRAGMENT ANTIBODIES IN THE INFANT RAT MODEL OF BACTEREMIA[1]

Rabbit antisera	cfu/0.1 mL blood		p value
	Pre-immune	Post-immunization	
Rb#434	510 (6/6)[2]	6 (1/6)	<0.001
Rb#435	910 (4/4)	6 (1/4)	<0.001

[1] Five-day old infant rats were passively immunized with 0.15 mL of rabbit anti-N-terminal rD15 fragment s.c. One day later, the infant rats were challenged with 200 cfu of H. influenzae type b strain MinnA (0.1 mL, IP). The blood samples were taken from each rat 24 hours after the challenge and analysed for bacteria counts.

[2] The parentheses indicate the number of rats found to be bacteremic out of the total number of rats challenged.

TABLE 2

SEQUENCE OF OVERLAPPING SYNTHETIC PEPTIDES ENCOMPASSING THE ENTIRE D15 ANTIGEN SEQUENCE

PEPTIDES	RESIDUES	SEQUENCES	SEQ ID NO:
D15-P1	20-49	APFVAKDIRVDGVQGDLEQQIRASLPVRAG	14
D15-P2	45-74	PVRAGQRVTDNDVAMIVRSLFVSGRFDDVK	15
D15-P3	68-99	GRFDDVKAHQEGDVLVVSVVAKSIISDVKIKG	16
D15-P4	93-122	SDVKIKGNSVIPTEALKQNLDANGFKVGDV	17
D15-P5	114-143	ANGFKVGDVLIREKLNEFAKSVKEHYASVG	18
D15-P6	135-164	VKEHYASVGRYNATVEPIVNTLPNNRAEIL	19
D15-P7	157-187	PNNRAEILIQINEDDKAKLASLTFKGNESVS	20
D15-P8	180-209	FKGNESVSSSTLQEQMELQPDSWWKKLWGNK	21
D15-P9	199-228	PDSWWKLWGNKFEGAQFEKDLQSIRDYYLN	22
D15-P10	219-249	LQSIRDYYLNNGYAKAQITKTDVQLNDEKTK	23
D15-P11	241-270	VQLNDEKTKVNVTIDVNEGLQYDLRSARII	24
D15-P12	262-291	YDLRSARIIGNLGGMSAELEPLLSALHLND	25
D15-P13	282-312	PLLSALHLNDTFRRSDIADVENAIKAKLGER	26
D15-P14	304-333	AIKAKLGERGYGSATVNSVPDFDDANKTLA	27
D15-P15	325-354	FDDANKTLAITLVVDAGRRLTVRQLRFEGN	28
D15-P16	346-375	VRQLRFEGNTVSADSTLRQEMRQQEGTWYN	29
D15-P17	367-396	RQQEGTWYNSQLVELGKIRLDRTGFFETVE	30
D15-P18	390-416	GFFETVENRIDPINGSNDEVDVVYKVK	31
D15-P19	410-435	DVVYKVKERNTGSINFGIGYGTESGI	32
D15-P20	430-455	GTESGISYQASVKQDNFLGTGAAVSI	33
D15-P21	450-477	GAAVSIAGTKNDYGTSVNLGYTEPYFTK	34
D15-P22	471-497	TEPYFTKDGVSLGGNVFFENYDNSKSD	35
D15-P23	491-516	YDNSKSDTSSNYKRTTYGSNVTLGFP	36
D15-P24	511-538	VTLGFPVNENNSYYVGLGHTYNKISNF	37
D15-P25	532-559	YNKISNFALEYNRNLYIQSMKFKGNGIK	38
D15-P26	554-582	KGNGIKTNDFDFSFGWNYNSLNRGYFPTK	39
D15-P27	577-602	GYFPTKGVKASLGGRVTIPGSDNKYYK	40
D15-P28	596-625	SDNKYYKLSADVQGFYPLDRDHLWVVSAK	41

D15-P29	619-646	LWVVSAKASAGYANGFGNKRLPFYQTYT	42
D15-P30	641-666	FYQTYTAGGIGSLRGFAYGSIGPNAI	43
D15-P31	662-688	GPNAIYAEYGNGSGTGTFKKISSDVIG	44
D15-P32	681-709	KISSDVIGGNAIATASAELIVPTPFVSDK	45
D15-P33	705-731	FVSDKSQNTVRTSLFVDAASVWNTKWK	46
D15-P34	725-750	VWNTKWKSDKNGLESDVLKRLPDYGK	47
D15-P35	745-771	LPDYGKSSRIRASTGVGFQWQSPIGPL	48
D15-P36	769-798	GPLVFSYAKPIKKYENDDVEQFQFSIGGSF	49

TABLE 3

REACTIVITY OF RABBIT AND GUINEA PIG ANTI-N-TERMINAL rD15 FRAGMENT ANTISERA WITH D15 SYNTHETIC PEPTIDES

	Reactive Titers				
	Rabbit antisera		Guinea pig antisera		
Peptides	3434	435	858	859	860
D15-P1	400	1,600	6,400	6,400	6,400
D15-P2	1,600	<100	100	100	<100
D15-P3	400	<100	100	<100	<100
D15-P4	25,600	6,400	<100	<100	<100
D15-P5	6,400	400	1,600	25,600	400
D15-P6	1,600	6,400	400	6,400	6,400
D15-P7	6,400	1,600	25,600	25,600	25,600
D15-P8	6,400	6,400	25,600	409,600	409,600
D15-P9	<100	<100	400	1,600	1,600
D15-P10	<100	<100	400	6,400	<100

TABLE 4

INHIBITION OF ANTI-N-TERMINAL rD15 FRAGMENT ANTIBODY-INDUCED PROTECTION BY D15 PEPTIDES IN THE INFANT RAT MODEL OF BACTEREMIA

Group #	Antibody	cfu / 10 μL blood	cfu in each group/ cfu in group #4 (control) (%)
1	Anti-D15 Ab + PBS	60 ± 120 (3/7)	3
2	Anti-D15 Ab + peptides	300 ± 240 (6/7)	13
3	Anti-D15 Ab + rD15	1,520 ± 1,280 (7/7)	64
4	PBS + peptides	2,360 ± 1,200 (6/7)	100

One half mL of rabbit anti-N-terminal rD15 fragment antiserum (Anti-rD15 fragment Ab) was mixed with either nine D15 peptides (100 μg of peptides D15-P2 to D15-P10, See TABLE 2) or with 600 μg of N-terminal rD15 fragment at room temperature for 1 hr. Antiserum and peptides mixed with PBS were used as controls. Seven-day old infant rats were injected s.c. with 0.2 mL of the various preparations. After 24 h, the infant rats were challenged I.P. with 200 cfu of *H. influenzae* type b strain MinnA. The blood samples were taken at 24 h after the challenge. The numbers in parentheses indicate the number of animals that were bacteremic out of the total number of animals challenged. The level of bacteremia is expressed as the mean of values obtained from seven infant rats tested individually ± one standard deviation (SD).

TABLE 5.

INHIBITION OF THE IMMUNOPROTECTION ABILITY OF THE RABBIT ANTI-N-TERMINAL rD15 FRAGMENT ANTISERUM ABSORBED WITH D15 PEPTIDES (D15-P4 TO D15-P8) IN THE INFANT RAT MODEL OF BACTEREMIA

Group #	Antibody	cfu / 10 μL blood	cfu in each group/ cfu in group #3 (%)
1	rD15 Ab + PBS	220 ± 360 (3/6)	8
2	rD15 Ab + peptides	2,960 ± 560 (6/6)	106
3	PBS + peptides	2,800 ± 360 (6/6)	100

One half mL of rabbit anti-rD15 fragment antiserum (rD15 Ab) was mixed with five D15 peptides (peptides P4 to P8, 250 μg of each peptide) at room temperature for 1 hr. Antiserum and peptides diluted in PBS were used as controls. Seven-day old infant rats were injected s.c. with 0.2 mL of the indicated material. After 24 h, the infant rats were challenged I.P. with 200 cfu of *H. influenzae* type b strain MinnA. The blood samples were collected 24 h after challenge. The numbers in parentheses indicate the number of animals that were bacteremia out of the total number of animals challenged. The level of bacteremia is expressed as the mean of values obtained from six infant rats tested individually ± one SD.

TABLE 6

REACTIVITY OF RABBIT, GUINEA PIG AND MOUSE ANTI-rD15 ANTISERA WITH D15 PEPTIDES

Peptide	Reactive Titer[1] Rabbit[2]	Guinea Pig[3]	Mouse[4]
D15-P1	-	-	+
D15-P2	-	+++	+
D15-P3	-	-	+
D15-P4	+	+	+
D15-P5	-	-	+
D15-P6	-	+	+
D15-P7	-	-	+
D15-P8	-	++++	++++
D15-P9	-	-	+
D15-P10	-	-	+++
D15-P11	-	-	+++
D15-P12	-	-	+
D15-P13	-	-	+
D15-P14	+++	+	+
D15-P15	-	-	+
D15-P16	-	-	+
D15-P17	-	-	+
D15-P18	-	-	+
D15-P19	-	-	+
D15-P20	-	-	+
D15-P21	-	-	+
D15-P22	-	-	+
D15-P23	-	-	+
D15-P24	-	-	+
D15-P25	-	-	+
D15-P26	-	-	+++
D15-P27	-	+	+

TABLE 6 (continued)

D15-P28	-	-	+
D15-P29	-	-	+
D15-P30	-	-	+
D15-P31	-	-	+
D15-P32	-	-	+
D15-P33	-	-	+
D15-P34	-	-	+
D15-P35	-	-	+
D15-P36	++++	-	+

[1] The reactive titer is based on peptide-specific ELISAs. +, ++, +++, and ++++ represent reactive titers of animal antisera tested at 1/300, 1/1000, 1/2000, and 1/5000 dilutions, respectively; - means nonreactive.

[2] Titer represents the average value of two rabbit antisera raised against rD15.

[3] Titer represents the average value of two guinea pig antisera raised against rD15.

[4] Titer represents the average value of five mouse antisera raised against r15.

TABLE 7

T-CELL STIMULATORY ACTIVITY OF D15 PEPTIDES

Peptide	CYTOKINE RELEASE (pg/mL)[1] IL-2[2]	γ-IFN[3]	IL-4[4]
D15-P1	-	-	-
D15-P2	122	-	-
D15-P3	25	-	-
D15-P4	-	-	-
D15-P5	*742*	*38,000*	*13*
D15-P6	-	-	-
D15-P7	-	-	-
D15-P8	-	-	-
D15-P9	-	-	-
D15-P10	108	1,900	-
D15-P11	-	-	-
D15-P12	1,052	6,100	-
D15-P13	*105*	*6,200*	*56*
D15-P14	-	-	-
D15-P15	-	-	-
D15-P16	48	-	-
D15-P17	-	-	-
D15-P18	32	4,800	-
D15-P19	882	24,500	-
D15-P20	-	-	-
D15-P21	-	-	-
D15-P22	-	-	-
D15-P23	78	-	-
D15-P24	103	-	-
D15-P25	-	-	-
D15-P26	572	6,700	-
D15-P27	*274*	*7,505*	*68*

TABLE 7 (continued)

D15-P28	142	742	-
D15-P29	-	-	-
D15-P30	-	-	-
D15-P31	-	-	-
D15-P32	-	-	-
D15-P33	-	-	-
D15-P34	82	603	-
D15-P35	107	751	-
D15-P36	-	-	-

[1] Results are expressed as mean values of triplicate cultures. All standard deviations were less than 15%. Immunodominant Th1-cell epitopes are highlighted with bold and Th0-cell epitopes are in italics.

TABLE 8

RABBIT AND GUINEA PIG ANTIBODY RESPONSES TO D15 PEPTIDES

	Peptide-specific ELISAs	
	Reactive Titer[1]	
Immunogen	Rabbit[2]	Guinea Pig[3]
D15-P1	102,400	819,200
D15-P2	204,800	1,637,400
D15-P3	51,200	1,637,400
D15-P4	204,800	819,200
D15-P5	51,200	1,637,400
D15-P6	51,200	409,600
D15-P7	204,800	819,200
D15-P8	51,200	409,600
D15-P9	102,400	409,600
D15-P10	102,400	819,200
D15-P11	51,200	819,200
D15-P12	102,400	204,800
D15-P13	NT[4]	204,800
D15-P14	NT	409,600
D15-P15	NT	204,800
D15-P16	NT	819,200
D15-P17	NT	204,800
D15-P18	NT	312,500
D15-P19	NT	312,500
D15-P20	NT	62,500
D15-P21	NT	62,500
D15-P22	NT	12,500
D15-P23	NT	1,562,500
D15-P24	NT	312,500
D15-P25	NT	62,500

TABLE 8 (continued)

D15-P26	NT	500
D15-P27	NT	1,500
D15-P28	NT	1,250
D15-P29	NT	<500
D15-P30	NT	<500
D15-P31	NT	<500
D15-P32	NT	12,500
D15-P33	NT	12,500
D15-P34	NT	62,500
D15-P35	NT	1,250
D15-P36	NT	12,500

[1] The reactive titer is based on peptide-specific ELISAs. A titer below 500 indicates that the peptide is not immunogenic.

[2] Titers represent the average value of obtained for two rabbit antisera raised against the D15 peptide.

[3] Titers represent the average value obtained for two guinea pig antisera raised against the D15 peptide.

[4] NT: not tested.

TABLE 9

RABBIT IgG ANTIBODY RESPONSE TO D15-PRP CONJUGATE

Rabbit[1] #	Reactive Titer Against[2]			
	PRP		rD15	
	2 doses	3 doses	2 doses	3 doses
489-1	1,600	3,200	1,600	6,400
490-1	1,600	1,600	6,400	25,600

[1] Rabbits were immunized intramuscularly with rD15-PRP conjugates (5 to 50 μg PRP equivalent) mixed with 3 mg $ALPO_4$ per mL, followed by two booster doses (half amount of the same immunogen) at 2 week intervals.

[2] Reactive titres is based on PRP specific and D-15 specific ELISAs.

SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides purified and isolated nucleic acid molecules containing genes encoding the D15 outer membrane protein, the sequences of these genes and the derived amino acid sequences thereof. The invention also provides peptides corresponding to portions of the D35 outer membrane protein. In addition, the invention provides antibodies raised against D15 outer membrane protein, fragments and peptides. The genes, DNA sequences, antibodies and peptides are useful for diagnosis, immunization and the generation of diagnostic and immunological reagents. Vaccines based on expressed recombinant D35, portions thereof or peptides derived from the provided sequences can be prepared for prevention of H. influenzae disease. Modification are possible within the scope of the invention.

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CLAIMS

What we claim is:

1. A purified and isolated nucleic acid molecule, the molecule comprising at least a portion coding for a D15 outer membrane protein and having a DNA sequence selected from (a) the DNA sequence set forth in any one of Figures 1A to 1E or its complementary strand, and (b) DNA sequences which hybridize under stringent conditions to the DNA sequences defined in (a).

2. The molecule of claim 1 wherein said DNA sequences defined (b) have at least 90% sequence identity with the sequences defined in (a).

3. The molecule of claim 1 wherein said DNA sequences defined in (b) comprise the consensus sequence set forth in Figure 1F.

4. A recombinant plasmid adapted for transformation of a host, the recombinant plasmid comprising a plasmid vector into which has been inserted a DNA segment comprising at least an 18 bp fragment of a DNA molecule of claim 1, 2 or 3

5. The recombinant plasmid of claim 4 which is selected from plasmid DS-712-2-1 having ATCC accession number 75604 deposited November 4, 1993 and plasmid JB-1042-5-1 having ATCC accession number 75606 deposited November 4, 1993.

6. A recombinant vector adapted for transformation of a host cell, the recombinant vector comprising at least a DNA segment comprising at least an 18 bp fragment of a DNA molecule of claim 1, 2 or 3 and expression means operatively coupled to the DNA segment for expression of the gene product encoded thereby in the host cell.

7. The recombinant vector of claim 6 being plasmid DS-880-1-2 having ATCC accession number 75605 deposited November 4, 1993 and encoding the D15 gene product of H. influenzae SB33.

8. The recombinant vector of claim 6 wherein said DNA segment encodes a polypeptide of at least 6 residues.

9. The recombinant vector of claim 8 wherein said polypeptide is selected from those shown in Table 2.

10. The recombinant vector of claim 6, 7, 8 or 9 wherein said DNA segment consists of no more than the coding sequence for said D15 outer membrane protein.

11. The recombinant vector of claim 10, wherein the DNA segment further comprises a nucleic acid sequence encoding a leader sequence for export of said gene product from said host.

12. A purified and isolated protein encoded by the DNA fragment contained in the recombinant vector of claim 10 or 11.

13. A purified and isolated D15 outer membrane protein, or a portion thereof.

14. The protein of claim 13 wherein the D15 outer membrane protein is a Haemophilus D15 outer membrane protein.

15. The protein of claim 14 wherein the D15 outer membrane protein is a Haemophilus influenzae D15 outer membrane protein.

16. The protein of claim 15 wherein the Haemophilus influenzae is a type b Haemophilus influenzae strain.

17. The protein of claim 16 wherein the Haemophilus influenzae type b strain is selected from Ca, MinnA and Eagan strains.

18. The protein of claim 15 wherein the Haemophilus influenzae is a non-typeable Haemophilus influenzae strain.

19. The protein of claim 18 wherein the non-typeable Haemophilus influenzae strain is selected from PAK12085 and SB33 strains.

20. A synthetic peptide containing an amino acid sequence corresponding to the amino acid sequence of the protein or portion thereof claimed in any one of claims

11 to 19, or variant or mutant which retains immunogenicity.

21. The peptide of claim 20, having an amino acid sequence selected from those contained in Table 2.

22. An immunogenic composition, comprising a nucleic acid molecule claimed ni any one of claims 1 to 5, a protein as claimed in any one of claims 12 to 14 or a peptide as claimed in claim 20 or 21 and a physiologically-acceptable carrier therefor.

23. The immunogenic composition of claim 22 formulated as a vaccine for in vivo administration to protect against diseases caused by Haemophilus.

24. The immunogenic composition of claim 23 formulated as a microparticle preparation, capsule preparation or liposome preparation.

25. The immunogenic composition of claim 23 in combination with a targeting molecule for delivery to specific cells of the immune system or to mucosal surfaces.

26. A method for inducing protection against disease caused by Haemophilus, comprising administering to a subject an effective amount of the immunogenic composition of any one of claims 22 to 25 to provide protective immunity against Haemophilus disease.

27. An antiserum or an antibody specific for a protein, a peptide or an immunogenic composition of any one of claims 12 to 25.

28. A chimeric molecule, comprising a protein as claimed in any one of claims 12 to 19 or a peptide claimed in claims 20 to 21 linked to another polypeptide or protein or a polysaccharide.

29. The chimeric molecule of claim 28 wherein said another polypeptide or protein comprise a surface protein or peptide corresponding thereto from a pathogenic bacteria.

30. The chimeric molecule of claim 29 wherein said another polypeptide or protein comprises a P1, P2 or P6 outer membrane protein of H. influenzae.

31. The chimeric molecule of claim 28 wherein said polysaccharide comprises a PRP molecule from H. influenzae.

FIG.1A.

H. influenzae b Ca strain D15 sequence

```
                    Hind III                    -35                                          -10
GATTACGCCAAGCTTAACGGTGTTTGCATTATTTAATGATTTTTTACGTCTATAATTTAT
          10                  20                  30                  40                  50                  60

     RBS                  MET  LYS  LYS  LEU  LEU  ILE  ALA  SER  LEU  LEU  PHE  GLY  THR  THR  THR  T
ATAGGATACAATCGATGAAAAAACTTCTAATCGCAAGTTTATTATTCGGTACGACAACGA
          70                  80                  90                  100                 110                 120
start truncated GST/D15 ──────┐
HR  VAL  PHE  ALA  ALA  PRO ↓ PHE  VAL  ALA  LYS  ASP  ILE  ARG  VAL  ASP  GLY  VAL  GLN  GLY  ASP  L
CTGTGTTTGCCGCACCTTTTGTGGCAAAAGATATTCGTGTGGATGGTGTTCAAGGTGACT
          130                 140                 150                 160                 170                 180

EU  GLU  GLN  GLN  ILE  ARG  ALA  SER  LEU  PRO  VAL  ARG  ALA  GLY  GLN  ARG  VAL  THR  ASP  ASN  A
TAGAACAACAAATCCGAGCAAGTTTACCTGTTCGTGCCGGTCAGCGTGTGACTGACAATG
          190                 200                 210                 220                 230                 240
                         spurious thrombin site
SP  VAL  ALA  ASN  ILE  VAL  ARG | SER  LEU  PHE  VAL  SER  GLY  ARG  PHE  ASP  ASP  VAL  LYS  ALA  H
ATGTGGCTAATATTGTCCGCTCTTTATTCGTAAGTGGTCGATTCGATGATGTGAAAGCGC
          250                 260                 270                 280                 290                 300

IS  GLN  GLU  GLY  ASP  VAL  LEU  VAL  VAL  SER  VAL  VAL  ALA  LYS  SER  ILE  ILE  SER  ASP  VAL  L
ATCAAGAAGGCGATGTGCTTGTTGTTAGCGTTGTGGCTAAATCGATCATTTCAGATGTTA
          310                 320                 330                 340                 350                 360
```

FIG.1A.(CONTINUED)

```
YS  ILE LYS GLY ASN SER VAL ILE PRO THR GLU ALA LEU LYS GLN ASN LEU ASP ALA ASN G
AAATCAAAGGTAACTCTGTTATTCCCACTGAAGCACTTAAACAAAACTTAGATGCTAACG
       370       380       390       400       410       420

LY  PHE LYS VAL GLY ASP VAL LEU ILE ARG GLU LYS LEU ASN GLU PHE ALA LYS SER VAL L
GGTTTAAAGTTGGCGATGTTTTAATTCGAGAAAAATTAAATGAATTTGCCAAAAGTGTAA
       430       440       450       460       470       480

YS  GLU HIS TYR ALA SER VAL GLY ARG TYR ASN ALA THR VAL GLU PRO ILE VAL ASN THR L
AAGAGCACTATGCAAGTGTAGGTCGCTATAACGCAACAGTTGAACCTATTGTCAATACGC
       490       500       510       520       530       540

EU  PRO ASN ASN ARG ALA GLU ILE LEU ILE GLN ILE ASN GLU ASP ASP LYS ALA LYS LEU A
TACCAAATAATCGCGCTGAAATTTTAATTCAAATCAATGAAGATGATAAAGCAAAATTGG
       550       560       570       580       590       600

LA  SER LEU THR PHE LYS GLY ASN GLU SER VAL SER SER SER THR LEU GLN GLU GLN MET G
CATCATTAACTTTCAAGGGGAACGAATCTGTTAGTAGCAGTACATTACAAGAACAAATGG
       610       620       630       640       650       660

LU  LEU GLN PRO ASP SER TRP TRP LYS LEU TRP GLY ASN LYS PHE GLU GLY ALA GLN PHE G
AATTACAACCTGATTCTTGGTGGAAATTATGGGGAAATAAATTTGAAGGTGCGCAATTCG
       670       680       690       700       710       720
  end truncated GST/D15 ---------+
                                 v
LU  LYS_ASP LEU GLN SER ILE ARG ASP TYR TYR LEU ASN ASN GLY TYR ALA LYS ALA GLN I
AGAAAGATTTGCAGTCAATTCGTGATTATTATTTAAATAATGGCTATGCCAAAGCACAAA
       730       740       750       760       770       780
```

FIG.1A.(CONTINUED)

```
LE  THR  LYS  THR  ASP  VAL  GLN  LEU  ASN  ASP  GLU  LYS  THR  LYS  VAL  ASN  VAL  THR  ILE  ASP  V
T T A C T A A A A C G G A T G T T C A G C T A A A T G A T G A A A A A A A C A A A A G T T A A T G T A A C C A T T G A T G
                790                 800                 810                 820                 830                 840

AL  ASN  GLU  GLY  LEU  GLN  TYR  ASP  LEU  ARG  SER  ALA  ARG  ILE  ILE  GLY  ASN  LEU  GLY  GLY  M
T A A A T G A A G G T T A C A G T A T G A C C T T C G T A G T G C A C G G C A T T A T A G G T A A T C T G G G A G G T A
                850                 860                 870                 880                 890                 900

ET  SER  ALA  GLU  LEU  GLU  PRO  LEU  LEU  SER  ALA  LEU  HIS  LEU  ASN  ASP  THR  PHE  ARG  ARG  S
T G T C T G C C G A G C T T G A A C C T T T A C T T T C A G C A T T T A C A T T T A A A T G A T A C T T T C C G C C G T A
                910                 920                 930                 940                 950                 960

ER  ASP  ILE  ALA  ASP  VAL  GLU  ASN  ALA  ILE  LYS  ALA  LYS  LEU  GLY  GLU  ARG  GLY  TYR  GLY  S
G T G A T A T T G C A G A T G T A G A A A A T G C A A T T A A A G C A A A A C T T G G A G A A C G C G G G T T A C G G T A
                970                 980                 990                1000                1010                1020

ER  ALA  THR  VAL  ASN  SER  VAL  PRO  ASP  PHE  ASP  ALA  ASN  LYS  THR  LEU  ALA  ILE  THR  L
G C G C A A C G G T A A A T T C A G T A C C T G A T T T T T G A T G C A A A A T A A A A C A T T A G C G A T A A C C C
                1030                1040                1050                1060                1070                1080

EU  VAL  ASP  ALA  GLY  ARG  LEU  THR  VAL  ARG  GLN  LEU  ARG  PHE  GLU  GLY  ASN  THR  V
T T G T T G T T G A T G C T G G A C G A C G T T T A A C T G T T C G G C C A A C T T C G C T T T G A A G G A A A T A C C G
                1090                1100                1110                1120                1130                1140
```

FIG.1A.(CONTINUED)

```
AL  SER  ALA  ASP  SER  THR  LEU  ARG  GLN  GLU  MET  ARG  GLN  GLN  GLU  GLY  THR  TRP  TYR  ASN  S
T T T C T G C T G A T A G C A C T T T A C G T C A G G A A A T G C G C C A A C A A G A A G G A A C T T G G T A T A A T T
              1150              1160              1170              1180              1190              1200

ER  GLN  LEU  VAL  GLU  LEU  GLY  LYS  ILE  ARG  LEU  ASP  ARG  THR  GLY  PHE  PHE  GLU  THR  VAL  G
C A C A A T T A G T T G A G T T A G G A A A A A T T C G C T T A G A T C G T A C A G G T T T C T T C G A A A C A G T C G
              1210              1220              1230              1240              1250              1260

LU  ASN  ARG  ILE  ASP  PRO  ILE  ASN  GLY  SER  ASN  ASP  GLU  VAL  ASP  VAL  VAL  TYR  LYS  VAL  L
A A A A C C G A A T T G A T C C T A T C A A T G G T A G T A A T G A T G A A G T G G A T G T C G T A T A T A A A G T C A
              1270              1280              1290              1300              1310              1320

YS  GLU  ARG  ASN  THR  GLY  SER  ILE  ASN  PHE  GLY  ILE  GLY  TYR  GLY  THR  GLU  SER  GLY  ILE  S
A A G A A C G T A A C A C G G G T A G T A T C A A C T T T G G T A T T G G T T A C G G T A C A G A G A G T G G T A T T A
              1330              1340              1350              1360              1370              1380

ER  TYR  GLN  ALA  SER  VAL  LYS  GLN  ASP  ASN  PHE  LEU  GLY  THR  GLY  ALA  ALA  VAL  SER  ILE  A
G T T A T C A A G C A A G T G T T A A A C A A G A T A A T T T C T T G G G A A C A G G G G C G G C A G T A A G T A T A G
              1390              1400              1410              1420              1430              1440

LA  GLY  THR  LYS  ASN  ASP  TYR  GLY  THR  SER  VAL  ASN  LEU  GLY  TYR  THR  GLU  PRO  TYR  PHE  T
C T G G T A C G A A A A A T G A T T A T G G T A C G A G T G T C A A T T T G G G T T A T A C C G A G C C C T A T T T T A
              1450              1460              1470              1480              1490              1500

HR  LYS  ASP  GLY  VAL  SER  LEU  GLY  GLY  ASN  VAL  PHE  PHE  GLU  ASN  TYR  ASP  ASN  SER  LYS  S
C T A A A G A T G G T G T A A G T C T T G G T G G A A A T G T T T T C T T T G A A A A C T A C G A T A A C T C T A A A A
              1510              1520              1530              1540              1550              1560
```

FIG.1A.(CONTINUED)

```
ER  ASP  THR  SER  SER  ASN  TYR  LYS  ARG  THR  THR  TYR  GLY  SER  ASN  VAL  THR  LEU  GLY  PHE  P
G T G A T A C A T C C T C T A A C T A T A A G C G T A C G A C T T A C G G A A G T A A T G T T A C T T T A G G T T T C C
              1570                 1580                 1590                 1600                 1610                 1620

RO  VAL  ASN  GLU  ASN  ASN  SER  TYR  TYR  VAL  GLY  LEU  GLY  HIS  THR  TYR  ASN  LYS  ILE  SER  A
C T G T A A A T G A A A A T A A C T C C T A T T A T G T A G G A T T A G G T C A T A C C T A T A A T A A A A T T A G T A
              1630                 1640                 1650                 1660                 1670                 1680

SN  PHE  ALA  LEU  GLU  TYR  ASN  ARG  ASN  LEU  TYR  ILE  GLN  SER  MET  LYS  PHE  LYS  GLY  ASN  G
A C T T T G C T C T A G A A T A T A A C C G T A A T T T A T A T A T T C A A T C A A T G A A A T T T A A A G G T A A T G
              1690                 1700                 1710                 1720                 1730                 1740

LY  ILE  LYS  THR  ASN  ASP  PHE  ASP  PHE  SER  PHE  GLY  TRP  ASN  TYR  ASN  SER  LEU  ASN  ARG  G
G C A T T A A A A C A A A T G A C T T T G A T T T T T C T T T T G G T T G G A A C T A T A A C A G C C T T A A T A G A G
              1750                 1760                 1770                 1780                 1790                 1800

LY  TYR  PHE  PRO  THR  LYS  GLY  VAL  LYS  ALA  SER  LEU  GLY  GLY  ARG  VAL  THR  ILE  PRO  GLY  S
G C T A T T T C C C A A C T A A A G G G G T T A A A G C A A G T C T T G G T G G A C G A G T T A C T A T T C C A G G T T
              1810                 1820                 1830                 1840                 1850                 1860

ER  ASP  ASN  LYS  TYR  TYR  LYS  LEU  SER  ALA  ASP  VAL  GLN  GLY  PHE  TYR  PRO  LEU  ASP  ARG  A
C T G A T A A C A A A T A C T A C A A A C T A A G T G C A G A T G T A C A G G G T T T C T A C C C A T T A G A C A G A G
              1870                 1880                 1890                 1900                 1910                 1920

SP  HIS  LEU  TRP  VAL  VAL  SER  ALA  LYS  ALA  SER  ALA  GLY  TYR  ALA  ASN  GLY  PHE  GLY  ASN  L
A T C A C C T C T G G G T T G T A T C T G C A A A A G C A T C T G C A G G A T A T G C A A A T G G T T T T G G A A A C A
              1930                 1940                 1950                 1960                 1970                 1980
```

FIG.1A.(CONTINUED)

YS ARG LEU PRO PHE TYR GLN THR TYR THR ALA GLY GLY ILE GLY SER LEU ARG GLY PHE A
A G C G T T T A C C G T T C T A T C A A A C T T A T A C A G C G G G T G G C A T C G G T T C A T T A C G T G G T T T T G
1990 2000 2010 2020 2030 2040

LA TYR GLY SER ILE GLY PRO ASN ALA ILE TYR ALA GLU TYR GLY ASN GLY SER GLY THR G
C T T A T G G T A G T A T T G G A C C T A A C G C A A T T T A T G C C G A A T A T G G T A A T G G T A G T G G T A C T G
2050 2060 2070 2080 2090 2100

LY THR PHE LYS LYS ILE SER SER ASP VAL ILE GLY GLY ASN ALA ILE ALA THR ALA SER A
G T A C T T T T A A G A A G A T A A G T T C T G A T G T G A T T G G T G G T A A T G C A A T C G C T A C A G C T A G C G
2110 2120 2130 2140 2150 2160

LA GLU LEU ILE VAL PRO THR PRO PHE VAL SER ASP LYS SER GLN ASN THR VAL ARG THR S
C A G A G T T A A T T G T G C C A A C T C C A T T T G T G A G C G A T A A G A G C C A A A A T A C G G T C C G A A C C T
2170 2180 2190 2200 2210 2220

ER LEU PHE VAL ASP ALA ALA SER VAL TRP ASN THR LYS TRP LYS SER ASP LYS ASN GLY L
C C T T A T T T G T T G A T G C G G C A A G T G T T T G G A A T A C T A A A T G G A A A T C A G A T A A A A A T G G A T
2230 2240 2250 2260 2270 2280

EU GLU SER ASP VAL LEU LYS ARG LEU PRO ASP TYR GLY LYS SER SER ARG ILE ARG ALA S
T A G A G A G C G A T G T A T T A A A A A G A T T G C C T G A T T A T G G C A A A T C A A G C C G T A T T C G C G C C T
2290 2300 2310 2320 2330 2340

FIG.1A.(CONTINUED)

```
ER  THR  GLY  VAL  GLY  PHE  GLN  TRP  GLN  SER  PRO  ILE  GLY  PRO  LEU  VAL  PHE  SER  TYR  ALA  L
C T A C A G G T G T C G G A T T C C A A T G G C A A T C T C C T A T T G G G C C A T T G G T A T T C T C T T A T G C C A
          2350              2360              2370              2380              2390              2400

YS  PRO  ILE  LYS  LYS  TYR  GLU  ASN  ASP  ASP  VAL  GLU  GLN  PHE  GLN  PHE  SER  ILE  GLY  GLY  S
A A C C A A T T A A A A A A T A T G A A A A T G A T G A T G T C G A A C A G T T C C A A T T T A G T A T T G G A G G T T
          2410              2420              2430              2440              2450              2460

ER  PHE  ***  ***
C T T T C T A A T A A A T T G A A C T T T T T T C T T C A T C A G A A C T C A A A A A C A A C G T T C T C T G C C T A A
          2470              2480              2490              2500              2510              2520

T T T A A T T G G G C A G A G A A A A T A T T A A A C C C A T C A T T T A A T T A A G G A T A T T T A T C A A A T G A A
          2530              2540              2550              2560              2570              2580

A A A C A T C G C A A A A G T A A C C G C A C T T G C T T T A G G T A T T G C A C T T G C T T C A G G C T A T G C T T C
          2590              2600              2610              2620              2630              2640

C G C T G A A G A A A A A A T T G C T T T C A T T A A T G C A G G T A T A T T T T T C A A C A T C A C C C A G A T C G C
          2650              2660              2670              2680              2690              2700

C A A G C G G T A G C A G A T A A A C T T G A T G C T G A A T T T A A A C C T G T A G C T G A G A A A T T A G C A G C A
          2710              2720              2730              2740              2750              2760
```

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FIG.1A.(CONTINUED)

```
AGCAAAAAAGAAAGTTGATGATAAAAATTGCTGCTCGTAAAAAAAGTAGAAGCAAAAAGTT
      2770      2780      2790      2800      2810      2820

GCGGCTTTAGAAAAAGATGCACCTCGCTTACGTCAAGCTGATATTCAAAAAACGCCCAACAG
      2830      2840      2850      2860      2870      2880

GAGATTAAATAAAATTAGGTGCGGCTGAAGATGCTGAATTACAAAAATTAATGCAAGAACAA
      2890      2900      2910      2920      2930      2940

GATAAAAAAA
```

FIG.1B.

DS-712-2-1 DNA, Eagan D15 sequence
IS THE SEQUENCE BEING TRANSLATED

```
A C A G G A C A G C T T T C C C T T T T A A C C T T G A A A A T A T T A G G G A A A T T A C T T C C T G G C G A T T T G
                10                  20                  30                  40                  50                  60

T C A T T A A A T A A T T T A A G T G G G C C A A T T T C T A T T G C A A A A G G T G C T G G C C C A T C A G C A A A T
                70                  80                  90                 100                 110                 120

A T T G G A T T G G T G T A T T T T T T A A G T T T T A T G G C A C T G A T T A G T G T A A A T T T A G G G A T T A T G
               130                 140                 150                 160                 170                 180

A A T T T A T T T C C A T T A C C A G T A T T A G A T G G C G G T C A T T T A G T T T T T T T T A A C A A T G G A A G C T
               190                 200                 210                 220                 230                 240

G T T A A A G G A A A A C C T G T T T C T G A G C G G G T G C A A A G C A T C T G T T A T C G A A T T G G C G C A G C A
               250                 260                 270                 280                 290                 300

C T G T T A T T A A G C T T A A C G G T G T T T G C A T T A T T T A A T G A T T T T T T A C G T C T A T A A T T T A T A
               310                 320                 330                 340                 350                 360
```


PCT/CA93/00501

FIG.1B.(CONTINUED)

```
                        MET  LYS  LYS  LEU  LEU  ILE  ALA  SER  LEU  LEU  PHE  GLY  THR  THR  THR  THR
TAGGATACAATCGATGAAAAAACTTCTAATCGCAAGTTTATTATTCGGTACGACAACGAC
          370            380            390            400            410            420

   VAL  PHE  ALA  ALA  PRO  PHE  VAL  ALA  LYS  ASP  ILE  ARG  VAL  ASP  GLY  VAL  GLN  GLY  ASP  LEU
TGTGTTTGCCGCACCTTTTGTGGCAAAAGATATTCGTGTGGATGGTGTTCAAGGTGACTT
          430            440            450            460            470            480

   GLU  GLN  GLN  ILE  ARG  ALA  SER  LEU  PRO  VAL  ARG  ALA  GLY  GLN  ARG  VAL  THR  ASP  ASN  ASP
AGAACAACAAATCCGAGCAAGTTTACCTGTTCGTGCCGGTCAGCGTGTGACTGACAATGA
          490            500            510            520            530            540

   VAL  ALA  ASN  ILE  VAL  ARG  SER  LEU  PHE  VAL  SER  GLY  ARG  PHE  ASP  ASP  VAL  LYS  ALA  HIS
TGTGGCTAATATTGTCCGCTCTTTATTCGTAAGTGGTCGATTCGATGATGTGAAAGCGCA
          550            560            570            580            590            600

   GLN  GLU  GLY  ASP  VAL  LEU  VAL  VAL  SER  VAL  VAL  ALA  LYS  SER  ILE  ILE  SER  ASP  VAL  LYS
TCAAGAAGGCGATGTGCTTGTTGTTAGCGTTGTGGCTAAATCGATCATTTCAGATGTTAA
          610            620            630            640            650            660

   ILE  LYS  GLY  ASN  SER  VAL  ILE  PRO  THR  GLU  ALA  LEU  LYS  GLN  ASN  LEU  ASP  ALA  ASN  GLY
AATCAAAAGGTAACTCTGTTATTCCCACTGAAGCACTTAAACAAAACTTAGATGCTAACGG
          670            680            690            700            710            720
```

FIG.1B.(CONTINUED)

```
   PHE  LYS  VAL  GLY  ASP  VAL  LEU  ILE  ARG  GLU  LYS  LEU  ASN  GLU  PHE  ALA  LYS  SER  VAL  LYS
G T T T A A A G T T G G C G A T G T T T T A A T T C G A G A A A A A T T A A A T G A A T T T G C C A A A A G T G T A A A
          730                  740                  750                  760                  770                  780

   GLU  HIS  TYR  ALA  SER  VAL  GLY  ARG  TYR  ASN  ALA  THR  VAL  GLU  PRO  ILE  VAL  ASN  THR  LEU
A G A G C A C T A T G C A A G T G T A G G T C G C T A T A A C G C A A C A G T T G A A C C T A T T G T C A A T A C G C T
          790                  800                  810                  820                  830                  840

   PRO  ASN  ASN  ARG  ALA  GLU  ILE  LEU  ILE  GLN  ILE  ASN  GLU  ASP  ASP  LYS  ALA  LYS  LEU  ALA
A C C A A A T A A T C G C G C T G A A A T T T T A A T T C A A A T C A A T G A A G A T G A T A A A G C A A A A T T G G C
          850                  860                  870                  880                  890                  900

   SER  LEU  THR  PHE  LYS  GLY  ASN  GLU  SER  VAL  SER  SER  SER  THR  LEU  GLN  GLU  GLN  MET  GLU
A T C A T T A A C T T T C A A G G G G A A C G A A T C T G T T A G T A G C A G T A C A T T A C A A G A A C A A A T G G A
          910                  920                  930                  940                  950                  960

   LEU  GLN  PRO  ASP  SER  TRP  TRP  LYS  LEU  TRP  GLY  ASN  LYS  PHE  GLU  GLY  ALA  GLN  PHE  GLU
A T T A C A A C C T G A T T C T T G G T G G A A A T T A T G G G G A A A T A A A T T T G A A G G T G C G C A A T T C G A
          970                  980                  990                 1000                 1010                 1020

   LYS  ASP  LEU  GLN  SER  ILE  ARG  ASP  TYR  TYR  LEU  ASN  ASN  GLY  TYR  ALA  LYS  ALA  GLN  ILE
G A A A G A T T T G C A G T C A A T T C G T G A T T A T T A T T T A A A T A A T G G C T A T G C C A A A G C A C A A A T
         1030                 1040                 1050                 1060                 1070                 1080

   THR  LYS  THR  ASP  VAL  GLN  LEU  ASN  ASP  GLU  LYS  THR  LYS  VAL  ASN  VAL  THR  ILE  ASP  VAL
T A C T A A A A C G G A T G T T C A G C T A A A T G A T G A A A A A A C A A A A G T T A A T G T A A C C A T T G A T G T
         1090                 1100                 1110                 1120                 1130                 1140
```

FIG.1B.(CONTINUED)

```
   ASN  GLU  GLY  LEU  GLN  TYR  ASP  LEU  ARG  SER  ALA  ARG  ILE  ILE  GLY  ASN  LEU  GLY  GLY  MET
AAATGAAGGTTTACAGTATGACCTTCGTAGTGCACGCATTATAGGTAATCTGGGAGGTAT
          1150         1160         1170         1180         1190         1200

   SER  ALA  GLU  LEU  GLU  PRO  LEU  LEU  SER  ALA  LEU  HIS  LEU  ASN  ASP  THR  PHE  ARG  ARG  SER
GTCTGCCGAGCTTGAACCTTTACTTTCAGCATTACATTTAAATGATACTTTCCGCCGTAG
          1210         1220         1230         1240         1250         1260

   ASP  ILE  ALA  ASP  VAL  GLU  ASN  ALA  ILE  LYS  ALA  LYS  LEU  GLY  GLU  ARG  GLY  TYR  GLY  SER
TGATATTGCAGATGTAGAAAATGCAATTAAAGCAAAACTTGGAGAACGCGGTTACGGTAG
          1270         1280         1290         1300         1310         1320

   ALA  THR  VAL  ASN  SER  VAL  PRO  ASP  PHE  ASP  ASP  ALA  ASN  LYS  THR  LEU  ALA  ILE  THR  LEU
CGCAACGGTAAATTCAGTACCTGATTTTGATGATGCAAATAAAACATTAGCGATAACCCT
          1330         1340         1350         1360         1370         1380

   VAL  VAL  ASP  ALA  GLY  ARG  ARG  LEU  THR  VAL  ARG  GLN  LEU  ARG  PHE  GLU  GLY  ASN  THR  VAL
TGTTGTTGATGCTGGACGACGTTTAACTGTTCGCCAACTTCGCTTTGAAGGAAATACCGT
          1390         1400         1410         1420         1430         1440

   SER  ALA  ASP  SER  THR  LEU  ARG  GLN  GLU  MET  ARG  GLN  GLN  GLU  GLY  THR  TRP  TYR  ASN  SER
TTCTGCTGATAGCACTTTACGTCAGGAAATGCGCCAACAAGAAGGAACTTGGTATAATTC
          1450         1460         1470         1480         1490         1500
```

FIG.1B.(CONTINUED)

GLN LEU VAL GLU LEU GLY LYS ILE ARG LEU ASP ARG THR GLY PHE PHE GLU THR VAL GLU
A C A A T T A G T T G A G T T A G G A A A A A T T C G C T T A G A T C G T A C A G G T T T C T T C G A A A C A G T C G A
1510 1520 1530 1540 1550 1560

ASN ARG ILE ASP PRO ILE ASN GLY SER ASN ASP GLU VAL ASP VAL VAL TYR LYS VAL LYS
A A A C C G A A T T G A T C C T A T C A A T G G T A G T A A T G A T G A A G T G G A T G T C G T A T A T A A A G T C A A
1570 1580 1590 1600 1610 1620

GLU ARG ASN THR GLY SER ILE ASN PHE GLY ILE GLY TYR GLY THR GLU SER GLY ILE SER
A G A A C G T A A C A C G G G T A G T A T C A A C T T T G G T A T T G G T T A C G G T A C A G A G A G T G G T A T T A G
1630 1640 1650 1660 1670 1680

TYR GLN ALA SER VAL LYS GLN ASP ASN PHE LEU GLY THR GLY ALA ALA VAL SER ILE ALA
T T A T C A A G C A A G T G T T A A A C A A G A T A A T T T C T T G G G A A C A G G G G C G G C A G T A A G T A T A G C
1690 1700 1710 1720 1730 1740

GLY THR LYS ASN ASP TYR GLY THR SER VAL ASN LEU GLY TYR THR GLU PRO TYR PHE THR
T G G T A C G A A A A A T G A T T A T G G T A C G A G T G T C A A T T T G G G T T A T A C C G A G C C C T A T T T T A C
1750 1760 1770 1780 1790 1800

LYS ASP GLY VAL SER LEU GLY GLY ASN VAL PHE PHE GLU ASN TYR ASP ASN SER LYS SER
T A A A G A T G G T G T A A G T C T T G G T G G A A A T G T T T T C T T T G A A A A C T A C G A T A A C T C T A A A A G
1810 1820 1830 1840 1850 1860

FIG.1B.(CONTINUED)

```
  ASP  THR  SER  SER  ASN  TYR  LYS  ARG  THR  THR  TYR  GLY  SER  ASN  VAL  THR  LEU  GLY  PHE  PRO
T GA TA CA TC CT CT AA CT AT AA GC GT AC GA CT TA CG GA AG TA AT GT TA CT TT AG GT TT CC C
          1870           1880           1890           1900           1910           1920

  VAL  ASN  GLU  ASN  ASN  SER  TYR  TYR  VAL  GLY  LEU  GLY  HIS  THR  TYR  ASN  LYS  ILE  SER  ASN
T GT AA AT GA AA AT AA CT CC TA TT AT GT AG GA TT AG GT CA TA CC TA TA AT AA AA TT AG TA A
          1930           1940           1950           1960           1970           1980

  PHE  ALA  LEU  GLU  TYR  ASN  ARG  ASN  LEU  TYR  ILE  GLN  SER  MET  LYS  PHE  LYS  GLY  ASN  GLY
C TT TG CT CT AG AA TA TA AC CG TA AT TT AT AT AT TC AA TC AA TG AA AT TT AA AG GT AA TG G
          1990           2000           2010           2020           2030           2040

  ILE  LYS  THR  ASN  ASP  PHE  ASP  PHE  SER  PHE  GLY  TRP  ASN  TYR  ASN  SER  LEU  ASN  ARG  GLY
C AT TA AA AC AA AT GA CT TT GA TT TT TC TT TT GG TT GG AA CT AT AA CA GC CT TA AT AG AG G
          2050           2060           2070           2080           2090           2100

  TYR  PHE  PRO  THR  LYS  GLY  VAL  LYS  ALA  SER  LEU  GLY  GLY  ARG  VAL  THR  ILE  PRO  GLY  SER
C TA TT TC CC AA CT AA AG GG GT TA AA GC AA GT CT TG GT GG AC GA GT TA CT AT TC CA GG TT C
          2110           2120           2130           2140           2150           2160

  ASP  ASN  LYS  TYR  TYR  LYS  LEU  SER  ALA  ASP  VAL  GLN  GLY  PHE  TYR  PRO  LEU  ASP  ARG  ASP
T GA TA AC AA AT AC TA CA AA CT AA GT GC AG AT GT AC AG GG TT TC TA CC CA TT AG AC AG AG A
          2170           2180           2190           2200           2210           2220

  HIS  LEU  TRP  VAL  VAL  SER  ALA  LYS  ALA  SER  ALA  GLY  TYR  ALA  ASN  GLY  PHE  GLY  ASN  LYS
T CA CC TC TG GG TT GT AT CT GC AA AA GC AT CT GC AG GA TA TG CA AA TG GT TT TG GA AA CA A
          2230           2240           2250           2260           2270           2280
```

FIG.1B.(CONTINUED)

```
   ARG  LEU  PRO  PHE  TYR  GLN  THR  TYR  THR  ALA  GLY  GLY  ILE  GLY  SER  LEU  ARG  GLY  PHE  ALA
G C G T T T A C C G T T C T A T C A A A C T T A T A C A G C G G G T G G C A T C G G T T C A T T A C G T G G T T T T G C
          2290                 2300                 2310                 2320                 2330                 2340

   TYR  GLY  SER  ILE  GLY  PRO  ASN  ALA  ILE  TYR  ALA  GLU  TYR  GLY  ASN  GLY  SER  GLY  THR  GLY
T T A T G G T A G T A T T G G A C C T A A C G C A A T T T A T G C C G A A T A T G G T A A T G G T A G T G G T A C T G G
          2350                 2360                 2370                 2380                 2390                 2400

   THR  PHE  LYS  LYS  ILE  SER  SER  ASP  VAL  ILE  GLY  GLY  ASN  ALA  ILE  ALA  THR  ALA  SER  ALA
T A C T T T T A A G A A G A T A A G T T C T G A T G T G A T T G G T G G T A A T G C A A T C G C T A C A G C T A G C G C
          2410                 2420                 2430                 2440                 2450                 2460

   GLU  LEU  ILE  VAL  PRO  THR  PRO  PHE  VAL  SER  ASP  LYS  SER  GLN  ASN  THR  VAL  ARG  THR  SER
A G A G T T A A T T G T G C C A A C T C C A T T T G T G A G C G A T A A G A G C C A A A A T A C G G T C C G A A C C T C
          2470                 2480                 2490                 2500                 2510                 2520

   LEU  PHE  VAL  ASP  ALA  ALA  SER  VAL  TRP  ASN  THR  LYS  TRP  LYS  SER  ASP  LYS  ASN  GLY  LEU
C T T A T T T G T T G A T G C G G C A A G T G T T T G G A A T A C T A A A T G G A A A T C A G A T A A A A A T G G A T T
          2530                 2540                 2550                 2560                 2570                 2580

   GLU  SER  ASP  VAL  LEU  LYS  ARG  LEU  PRO  ASP  TYR  GLY  LYS  SER  SER  ARG  ILE  ARG  ALA  SER
A G A G A G C G A T G T A T T A A A A A G A T T G C C T G A T T A T G G C A A A T C A A G C C G T A T T C G C G C C T C
          2590                 2600                 2610                 2620                 2630                 2640
```

FIG.1B.(CONTINUED)

```
   THR  GLY  VAL  GLY  PHE  GLN  TRP  GLN  SER  PRO  ILE  GLY  PRO  LEU  VAL  PHE  SER  TYR  ALA  LYS
TACAGGTGTCGGATTCCAATGGCAATCTCCTATTGGGCCATTGGTATTCTCTTATGCCAA
           2650          2660          2670          2680          2690          2700

   PRO  ILE  LYS  LYS  TYR  GLU  ASN  ASP  ASP  VAL  GLU  GLN  PHE  GLN  PHE  SER  ILE  GLY  GLY  SER
ACCAATTAAAAAATATGAAAATGATGATGTCGAACAGTTCCAATTTAGTATTGGAGGTTC
           2710          2720          2730          2740          2750          2760

   PHE  ***  ***
TTTCTAATAAATTGAACTTTTTTCTTCATCAGAACTCAAAAACAACGTTCTCTGCCTAAT
           2770          2780          2790          2800          2810          2820

TTAATTGGGCAGAGAAAATATTAAACCCATCATTTAATTAAGGATATTTATCAAATGAAA
           2830          2840          2850          2860          2870          2880

AACATCGCAAAAGTAACCGCACTTGCTTTAGGTATTGCACTTGCTTCAGGCTATGCTTCC
           2890          2900          2910          2920          2930          2940

GCTGAAGAAAAAATTGCTTTCATTAATGCACTTATATTTTTCAA
           2950          2960          2970          2980
```

FIG.1C.

DS-691-1-5 DNA, Minn A D15 sequence
IS THE SEQUENCE BEING TRANSLATED

```
A A T C A C T T A C T G G C G A T T T G T C A T T A A A T A A T T T A A G T G G G C C A A T T T C T A T T G C A A A A G
                10                  20                  30                  40                  50                  60

G T G C T G G C A C A T C A G C A A A T A T T G G A T T G G T G T A T T T T T T A A G T T T T A T G G C A C T G A T T A
                70                  80                  90                 100                 110                 120

G T G T A A A T T T A G G G A T T A T G A A T T T A T T T C C A T T A C C A G T A T T A G A T G G C G G T C A T T T A G
               130                 140                 150                 160                 170                 180

T T T T T T T A A C A A T G G A A G C T G T T A A A G G A A A A C C T G T T T C T G A G C G G G T G C A A A G C A T C T
               190                 200                 210                 220                 230                 240

G T T A T C G A A T T G G C G C A G C A C T G T T A T T A A G C T T A A C G G T G T T T G C A T T A T T T A A T G A T T
               250                 260                 270                 280                 290                 300
```

FIG.1C.(CONTINUED)

```
                                                                  MET  LYS  LYS  LEU  LEU  ILE  ALA  SER  LEU
T T T T A C G T C T A T A A T T T A T A T A G G A T A C A A T C G A T G A A A A A A C T T C T A A T C G C A A G T T T A
              310                  320                  330                  340                  350                  360

 LEU  PHE  GLY  THR  THR  THR  THR  VAL  PHE  ALA  ALA  PRO  PHE  VAL  ALA  LYS  ASP  ILE  ARG  VAL
T T A T T C G G T A C G A C A A C G A C T G T G T T T G C C G C A C C T T T T G T G G C A A A A G A T A T T C G T G T G
              370                  380                  390                  400                  410                  420

 ASP  GLY  VAL  GLN  GLY  ASP  LEU  GLU  GLN  GLN  ILE  ARG  ALA  SER  LEU  PRO  VAL  ARG  ALA  GLY
G A T G G T G T T C A A G G T G A C T T A G A A C A A C A A A T C C G A G C A A G T T T A C C T G T T C G T G C C G G T
              430                  440                  450                  460                  470                  480

 GLN  ARG  VAL  THR  ASP  ASN  ASP  VAL  ALA  ASN  ILE  VAL  ARG  SER  LEU  PHE  VAL  SER  GLY  ARG
C A G C G T G T G A C T G A C A A T G A T G T G G C T A A T A T T G T C C G C T C T T T A T T C G T A A G T G G T C G A
              490                  500                  510                  520                  530                  540

 PHE  ASP  ASP  VAL  LYS  ALA  HIS  GLN  GLU  GLY  ASP  VAL  LEU  VAL  VAL  SER  VAL  VAL  ALA  LYS
T T C G A T G A T G T G A A A G C G C A T C A A G A A G G C G A T G T G C T T G T T G T T A G C G T T G T G G C T A A A
              550                  560                  570                  580                  590                  600

 SER  ILE  ILE  SER  ASP  VAL  LYS  ILE  LYS  GLY  ASN  SER  VAL  ILE  PRO  THR  GLU  ALA  LEU  LYS
T C G A T C A T T T C A G A T G T T A A A A T C A A A G G T A A C T C T G T T A T T C C C A C T G A A G C A C T T A A A
              610                  620                  630                  640                  650                  660
```

FIG.1C.(CONTINUED)

```
 GLN  ASN  LEU  ASP  ALA  ASN  GLY  PHE  LYS  VAL  GLY  ASP  VAL  LEU  ILE  ARG  GLU  LYS  LEU  ASN
C A A A A C T T A G A T G C T A A C G G G T T T A A A G T T G G C G A T G T T T T A A T T C G A G A A A A A T T A A A T
              670                  680                  690                  700                  710                  720

 GLU  PHE  ALA  LYS  SER  VAL  LYS  GLU  HIS  TYR  ALA  SER  VAL  GLY  ARG  TYR  ASN  ALA  THR  VAL
G A A T T T G C C A A A A G T G T A A A A G A G C A C T A T G C A A G T G T A G G T C G C T A T A A C G C A A C A G T T
              730                  740                  750                  760                  770                  780

 GLU  PRO  ILE  VAL  ASN  THR  LEU  PRO  ASN  ASN  ARG  ALA  GLU  ILE  LEU  ILE  GLN  ILE  ASN  GLU
G A A C C T A T T G T C A A T A C G C T A C C A A A T A A T C G C G C T G A A A T T T T A A T T C A A A T C A A T G A A
              790                  800                  810                  820                  830                  840

 ASP  ASP  LYS  ALA  LYS  LEU  ALA  SER  LEU  THR  PHE  LYS  GLY  ASN  GLU  SER  VAL  SER  SER  SER
G A T G A T A A A G C A A A A T T G G C A T C A T T A A C T T T C A A G G G G A A C G A A T C T G T T A G T A G T A G C A G T
              850                  860                  870                  880                  890                  900

 THR  LEU  GLN  GLU  GLN  MET  GLU  LEU  GLN  PRO  ASP  SER  TRP  TRP  LYS  LEU  TRP  GLY  ASN  LYS
A C A T T A C A A G A A C A A A T G G A A T T A C A A C C T G A T T C T T G G T G G A A A T T A T G G G G A A A T A A A
              910                  920                  930                  940                  950                  960

 PHE  GLU  GLY  ALA  GLN  PHE  GLU  LYS  ASP  LEU  GLN  SER  ILE  ARG  ASP  TYR  TYR  LEU  ASN  ASN
T T T G A A G G T G C G C A A T T C G A G A A A G A T T T G C A G T C A A T T C G T G A T T A T T A T T T A A A T A A T
              970                  980                  990                  1000                 1010                 1020
```

FIG.1C.(CONTINUED)

```
 GLY  TYR  ALA  LYS  ALA  GLN  ILE  THR  LYS  THR  ASP  VAL  GLN  LEU  ASN  ASP  GLU  LYS  THR  LYS
GGCTATGCCAAAGCACAAATTACTAAAACGGATGTTCAGCTAAATGATGAAAAAACAAAA
          1030          1040          1050          1060          1070          1080

 VAL  ASN  VAL  THR  ILE  ASP  VAL  ASN  GLU  GLY  LEU  GLN  TYR  ASP  LEU  ARG  SER  ALA  ARG  ILE
GTTAATGTAACCATTGATGTAAATGAAGGTTTACAGTATGACCTTCGTAGTGCACGCATT
          1090          1100          1110          1120          1130          1140

 ILE  GLY  ASN  LEU  GLY  GLY  MET  SER  ALA  GLU  LEU  GLU  PRO  LEU  LEU  SER  ALA  LEU  HIS  LEU
ATAGGTAATCTGGGAGGTATGTCTGCCGAGCTTGAACCTTTACTTTCAGCATTACATTTA
          1150          1160          1170          1180          1190          1200

 ASN  ASP  THR  PHE  ARG  ARG  SER  ASP  ILE  ALA  ASP  VAL  GLU  ASN  ALA  ILE  LYS  ALA  LYS  LEU
AATGATACTTTCCGCCGTAGTGATATTGCAGATGTAGAAAATGCAATTAAAGCAAAACTT
          1210          1220          1230          1240          1250          1260

 GLY  GLU  ARG  GLY  TYR  GLY  SER  ALA  THR  VAL  ASN  SER  VAL  PRO  ASP  PHE  ASP  ASP  ALA  ASN
GGAGAACGCGGTTACGGTAGCGCAACGGTAAATTCAGTACCTGATTTTGATGATGCAAAT
          1270          1280          1290          1300          1310          1320

 LYS  THR  LEU  ALA  ILE  THR  LEU  VAL  VAL  ASP  ALA  GLY  ARG  ARG  LEU  THR  VAL  ARG  GLN  LEU
AAAACATTAGCGATAACCCTTGTTGTTGATGCTGGACGACGTTTAACTGTTCGCCAACTT
          1330          1340          1350          1360          1370          1380
```

FIG.1C.(CONTINUED)

```
ARG  PHE  GLU  GLY  ASN  THR  VAL  SER  ALA  ASP  SER  THR  LEU  ARG  GLN  GLU  MET  ARG  GLN  GLN
CGCTTTGAAGGAAATACCGTTTCTGCTGATAGCACTTTACGTCAGGAAATGCGCCAACAA
          1390           1400           1410           1420           1430           1440

GLU  GLY  THR  TRP  TYR  ASN  SER  GLN  LEU  VAL  GLU  LEU  GLY  LYS  ILE  ARG  LEU  ASP  ARG  THR
GAAGGAACTTGGTATAATTCACAATTAGTTGAGTTAGGAAAAATTCGCTTAGATCGTACA
          1450           1460           1470           1480           1490           1500

GLY  PHE  PHE  GLU  THR  VAL  GLU  ASN  ARG  ILE  ASP  PRO  ILE  ASN  GLY  SER  ASN  ASP  GLU  VAL
GGTTTCTTCGAAACAGTCGAAAACCGAATTGATCCTATCAATGGTAGTAATGATGAAGTG
          1510           1520           1530           1540           1550           1560

ASP  VAL  VAL  TYR  LYS  VAL  LYS  GLU  ARG  ASN  THR  GLY  SER  ILE  ASN  PHE  GLY  ILE  GLY  TYR
GATGTCGTATATAAAGTCAAAGAACGTAACACGGGTAGTATCAACTTTGGTATTGGTTAC
          1570           1580           1590           1600           1610           1620

GLY  THR  GLU  SER  GLY  ILE  SER  TYR  GLN  ALA  SER  VAL  LYS  GLN  ASP  ASN  PHE  LEU  GLY  THR
GGTACAGAGAGTGGTATTAGTTATCAAGCAAGTGTTAAACAAGATAATTTCTTGGGAACA
          1630           1640           1650           1660           1670           1680

GLY  ALA  ALA  VAL  SER  ILE  ALA  GLY  THR  LYS  ASN  ASP  TYR  GLY  THR  SER  VAL  ASN  LEU  GLY
GGGGCGGCAGTAAGTATAGCTGGTACGAAAAATGATTATGGTACGAGTGTCAATTTGGGT
          1690           1700           1710           1720           1730           1740
```

FIG.1C.(CONTINUED)

```
 TYR  THR  GLU  PRO  TYR  PHE  THR  LYS  ASP  GLY  VAL  SER  LEU  GLY  GLY  ASN  VAL  PHE  PHE  GLU
T A T A C C G A G C C C T A T T T T A C T A A A G A T G G T G T A A G T C T T G G T G G A A A T G T T T T C T T T G A A
              1750                 1760                 1770                 1780                 1790                 1800

 ASN  TYR  ASP  ASN  SER  LYS  SER  ASP  THR  SER  SER  ASN  TYR  LYS  ARG  THR  THR  TYR  GLY  SER
A A C T A C G A T A A C T C T A A A A G T G A T A C A T C C T C T A A C T A T A A G C G T A C G A C T T A C G G A A G T
              1810                 1820                 1830                 1840                 1850                 1860

 ASN  VAL  THR  LEU  GLY  PHE  PRO  VAL  ASN  GLU  ASN  ASN  SER  TYR  TYR  VAL  GLY  LEU  GLY  HIS
A A T G T T A C T T T A G G T T T C C C T G T A A A T G A A A A T A A C T C C T A T T A T G T A G G A T T A G G T C A T
              1870                 1880                 1890                 1900                 1910                 1920

 THR  TYR  ASN  LYS  ILE  SER  ASN  PHE  ALA  LEU  GLU  TYR  ASN  ARG  ASN  LEU  TYR  ILE  GLN  SER
A C C T A T A A T A A A A T T A G T A A C T T T G C T C T A G A A T A T A A C C G T A A T T T A T A T A T T C A A T C A
              1930                 1940                 1950                 1960                 1970                 1980

 MET  LYS  PHE  LYS  GLY  ASN  GLY  ILE  LYS  THR  ASN  ASP  PHE  ASP  PHE  SER  PHE  GLY  TRP  ASN
A T G A A A T T T A A A G G T A A T G G C A T T A A A A C A A A T G A C T T T G A T T T T T C T T T T G G T T G G A A C
              1990                 2000                 2010                 2020                 2030                 2040

 TYR  ASN  SER  LEU  ASN  ARG  GLY  TYR  PHE  PRO  THR  LYS  GLY  VAL  LYS  ALA  SER  LEU  GLY  GLY
T A T A A C A G C C T T A A T A G A G G C T A T T T C C C A A C T A A A G G G G T T A A A G C A A G T C T T G G T G G A
              2050                 2060                 2070                 2080                 2090                 2100
```

FIG.1C.(CONTINUED)

```
ARG  VAL  THR  ILE  PRO  GLY  SER  ASP  ASN  LYS  TYR  TYR  LYS  LEU  SER  ALA  ASP  VAL  GLN  GLY
CGAGTTACTATTCCAGGTTCTGATAACAAATACTACAAACTAAGTGCAGATGTACAGGGT
           2110           2120           2130           2140           2150           2160

PHE  TYR  PRO  LEU  ASP  ARG  ASP  HIS  LEU  TRP  VAL  VAL  SER  ALA  LYS  ALA  SER  ALA  GLY  TYR
TTCTACCCATTAGACAGAGATCACCTCTGGGTTGTATCTGCAAAAGCATCTGCAGGATAT
           2170           2180           2190           2200           2210           2220

ALA  ASN  GLY  PHE  GLY  ASN  LYS  ARG  LEU  PRO  PHE  TYR  GLN  THR  TYR  THR  ALA  GLY  GLY  ILE
GCAAATGGTTTTGGAAACAAGCGTTTACCGTTCTATCAAACTTATACAGCGGGTGGCATC
           2230           2240           2250           2260           2270           2280

GLY  SER  LEU  ARG  GLY  PHE  ALA  TYR  GLY  SER  ILE  GLY  PRO  ASN  ALA  ILE  TYR  ALA  GLU  TYR
GGTTCATTACGTGGTTTTGCTTATGGTAGTATTGGACCTAACGCAATTTATGCCGAATAT
           2290           2300           2310           2320           2330           2340

GLY  ASN  GLY  SER  GLY  THR  GLY  THR  PHE  LYS  LYS  ILE  SER  SER  ASP  VAL  ILE  GLY  GLY  ASN
GGTAATGGTAGTGGTACTGGTACTTTTAAGAAGATAAGTTCTGATGTGATTGGTGGTAAT
           2350           2360           2370           2380           2390           2400

ALA  ILE  ALA  THR  ALA  SER  ALA  GLU  LEU  ILE  VAL  PRO  THR  PRO  PHE  VAL  SER  ASP  LYS  SER
GCAATCGCTACAGCTAGCGCAGAGTTAATTGTGCCAACTCCATTTGTGAGCGATAAGAGC
           2410           2420           2430           2440           2450           2460
```

FIG.1C.(CONTINUED)

```
 GLN  ASN  THR  VAL  ARG  THR  SER  LEU  PHE  VAL  ASP  ALA  ALA  SER  VAL  TRP  ASN  THR  LYS  TRP
C A A A A T A C G G T C C G A A C C T C C T T A T T T G T T G A T G C G G C A A G T G T T T G G A A T A C T A A A T G G
            2470              2480              2490              2500              2510              2520

 LYS  SER  ASP  LYS  ASN  GLY  LEU  GLU  SER  ASP  VAL  LEU  LYS  ARG  LEU  PRO  ASP  TYR  GLY  LYS
A A A T C A G A T A A A A A T G G A T T A G A G A G C G A T G T A T T A A A A A G A T T G C C T G A T T A T G G C A A A
            2530              2540              2550              2560              2570              2580

 SER  SER  ARG  ILE  ARG  ALA  SER  THR  GLY  VAL  GLY  PHE  GLN  TRP  GLN  SER  PRO  ILE  GLY  PRO
T C A A G C C G T A T T C G C G C C T C T A C A G G T G T C G G A T T C C A A T G G C A A T C T C C T A T T G G G C C A
            2590              2600              2610              2620              2630              2640

 LEU  VAL  PHE  SER  TYR  ALA  LYS  PRO  ILE  LYS  LYS  TYR  GLU  ASN  ASP  ASP  VAL  GLU  GLN  PHE
T T G G T A T T C T C T T A T G C C A A A C C A A T T A A A A A A T A T G A A A A T G A T G A T G T C G A A C A G T T C
            2650              2660              2670              2680              2690              2700

 GLN  PHE  SER  ILE  GLY  GLY  SER  PHE  ***  ***
C A A T T T A G T A T T G G A G G T T C T T T C T A A T A A A T T G A A C T T T T T T C T T C A T C A G A A C T C A A A
            2710              2720              2730              2740              2750              2760
```

FIG.1C.(CONTINUED)

```
AACAACGTTCTCTGCCTAATTTAATTGGGCAGAGAAAATATTAAACCCATCATTTAATTA
          2770       2780       2790       2800       2810       2820

AGGATATTTATCAAATGAAAAACATCGCAAAAGTAACCGCACTTGCTTTAGGTATTGCAC
          2830       2840       2850       2860       2870       2880

TTGCTTCAGGCTATGCTTCCGCTGAAGAAAAAATTGCTTTCATTAATGCGGGTTATANTT
          2890       2900       2910       2920       2930       2940

TNCAAGGCNAAGG
          2950
```

FIG.1D.

SB33 D15

IS THE SEQUENCE BEING TRANSLATED

```
GGCATTGAAAAAACAGGACAGCTTTCCCTTTTAACCTTGAAAATATTAGGGAAATTACTT
          10        20        30        40        50        60

ACTGGCGATTTGTCATTAAATAATTTAAGTGGGCCAATTTCTATTGCAAAAGGTGCTGGC
          70        80        90       100       110       120

GCATCAGCAAATATTGGATTGGTGTATTTTTTAAGTTTTATGGCATTGATTAGTGTAAAT
         130       140       150       160       170       180

TTAGGGATTATGAATTTATTTCCATTACCAGTATTAGATGGCGGTCATTTAGTTTTTTTA
         190       200       210       220       230       240

ACAATGGAAGCTGTTAAAGGAAAACCTGTTTCTGAGCGGGTGCAAAGCATCTGTTATCGA
         250       260       270       280       290       300
```

FIG.1D.(CONTINUED)

```
A T T G G C G C A G C A C T G T T A T T A A G C T T A A C G G T G T T T G C A T T A T T T A A T G A T T T T T T A C G T
              310                 320                 330                 340                 350                 360

                                                    MET  LYS  LYS  LEU  LEU  ILE  ALA  SER  LEU  LEU  PHE  GLY
C T A T A A T T T A T A T A G G A T A C A A T C G A T G A A A A A A C T T C T A A T C G C A A G T T T A T T A T T C G G
              370                 380                 390                 400                 410                 420

  THR  THR  THR  THR  VAL  PHE  ALA  ALA  PRO  PHE  VAL  ALA  LYS  ASP  ILE  ARG  VAL  ASP  GLY  VAL
T A C G A C A A C G A C T G T G T T T G C C G C A C C T T T T G T G G C A A A A G A T A T T C G T G T G G A T G G T G T
              430                 440                 450                 460                 470                 480

  GLN  GLY  ASP  LEU  GLU  GLN  GLN  ILE  ARG  ALA  SER  LEU  PRO  VAL  ARG  ALA  GLY  GLN  ARG  VAL
T C A A G G T G A C T T A G A A C A A C A A A T C C G A G C A A G T T T A C C T G T T C G T G C C G G T C A G C G T G T
              490                 500                 510                 520                 530                 540

  THR  ASP  ASN  ASP  VAL  ALA  ASN  ILE  VAL  ARG  SER  LEU  PHE  VAL  SER  GLY  ARG  PHE  ASP  ASP
G A C T G A C A A T G A T G T G G C T A A T A T T G T C C G C T C T T T A T T C G T A A G T G G T C G A T T C G A T G A
              550                 560                 570                 580                 590                 600

  VAL  LYS  ALA  HIS  GLN  GLU  GLY  ASP  VAL  LEU  VAL  VAL  SER  VAL  VAL  ALA  LYS  SER  ILE  ILE
T G T G A A A G C G C A T C A A G A A G G C G A T G T G C T T G T T G T T A G C G T T G T G G C T A A A T C G A T C A T
              610                 620                 630                 640                 650                 660
```

FIG.1D.(CONTINUED)

```
   SER  ASP  VAL  LYS  ILE  LYS  GLY  ASN  SER  ILE  ILE  PRO  PRO  GLU  ALA  LEU  LYS  GLN  ASN  LEU
TTCAGATGTTAAAATCAAAGGTAACTCTATTATTCCACCTGAAGCACTAAAACAAAACTT
       670          680          690          700          710          720

   ASP  ALA  ASN  GLY  PHE  LYS  VAL  GLY  ASP  ILE  LEU  ILE  ARG  GLU  LYS  LEU  ASN  GLU  PHE  ALA
AGATGCTAACGGGTTTAAAGTTGGCGATATTTTAATTCGAGAAAAATTAAATGAATTTGC
       730          740          750          760          770          780

   GLN  SER  VAL  LYS  GLU  HIS  TYR  ALA  SER  VAL  GLY  ARG  TYR  ASN  ALA  THR  VAL  GLU  PRO  ILE
CCAAAGTGTAAAAGAGCACTATGCAAGTGTAGGTCGCTATAACGCAACCGTTGAACCTAT
       790          800          810          820          830          840

   VAL  ASN  THR  LEU  PRO  ASN  ASN  ARG  ALA  GLU  ILE  LEU  ILE  GLN  ILE  ASN  GLU  ASP  ASP  LYS
TGTCAATACGCTACCAAATAATCGCGCTGAAATTTTAATTCAAATCAATGAAGATGATAA
       850          860          870          880          890          900

   ALA  LYS  LEU  ALA  SER  LEU  THR  PHE  LYS  GLY  ASN  GLU  SER  VAL  SER  SER  SER  THR  LEU  GLN
AGCCAAATTGGCATCATTAACTTTCAAGGGGAACGAATCTGTTAGTAGCAGTACATTACA
       910          920          930          940          950          960

   GLU  GLN  MET  GLU  LEU  GLN  PRO  ASP  SER  TRP  TRP  LYS  LEU  TRP  GLY  ASN  LYS  PHE  GLU  GLY
AGAACAAATGGAATTACAACCTGATTCTTGGTGGAAATTATGGGGAAATAAATTTGAAGG
       970          980          990          1000         1010         1020
```

FIG.1D.(CONTINUED)

```
   ALA  GLN  PHE  GLU  LYS  ASP  LEU  GLN  ALA  ILE  ARG  ASP  TYR  TYR  LEU  ASN  ASN  GLY  TYR  ALA
T G C G C A A T T C G A G A A A G A T T T G C A G G C A A T T C G T G A T T A T T A T T T A A A T A A T G G C T A T G C
             1030              1040              1050              1060              1070              1080

   LYS  ALA  GLN  ILE  THR  LYS  ALA  ASP  VAL  GLN  LEU  ASN  ASP  GLU  LYS  THR  LYS  VAL  ASN  VAL
C A A A G C A C A A A T C A C T A A A G C G G A T G T T C A G C T A A A T G A T G A A A A A A C A A A A G T T A A T G T
             1090              1100              1110              1120              1130              1140

   THR  ILE  ASP  VAL  ASN  GLU  GLY  LEU  GLN  TYR  ASP  LEU  ARG  SER  ALA  ARG  ILE  ILE  GLY  ASN
A A C C A T T G A T G T A A A T G A A G G T T T A C A G T A T G A C C T T C G T A G T G C A C G C A T T A T A G G T A A
             1150              1160              1170              1180              1190              1200

   LEU  GLY  GLY  MET  SER  ALA  GLU  LEU  GLU  PRO  LEU  LEU  SER  ALA  LEU  HIS  LEU  ASN  ASP  THR
T C T G G G A G G T A T G T C T G C C G A G C T T G A A C C T T T A C T T T C A G C A T T A C A T T T A A A T G A T A C
             1210              1220              1230              1240              1250              1260

   PHE  ARG  ARG  SER  ASP  ILE  ALA  ASP  VAL  GLU  ASN  ALA  ILE  LYS  ALA  LYS  LEU  GLY  GLU  ARG
T T T C C G C C G T A G T G A T A T T G C A G A T G T A G A A A A T G C A A T T A A A G C A A A A C T T G G G G A A C G
             1270              1280              1290              1300              1310              1320

   GLY  TYR  GLY  ASN  THR  THR  VAL  ASN  SER  VAL  PRO  ASP  PHE  ASP  ASP  ALA  ASN  LYS  THR  LEU
A G G T T A C G G T A A C A C A A C A G T A A A T T C T G T A C C T G A T T T T G A C G A T G C A A A T A A A A C A T T
             1330              1340              1350              1360              1370              1380
```

FIG.1D.(CONTINUED)

```
   ALA  ILE  THR  PHE  VAL  VAL  ASP  ALA  GLY  ARG  ARG  LEU  THR  VAL  HIS  GLN  LEU  ARG  PHE  GLU
A G C G A T A A C C T T T G T T G T T G A T G C T G G A C G A C G T T T A A C T G T T C A C C A A C T T C G C T T T G A
           1390              1400              1410              1420              1430              1440

   GLY  ASN  THR  VAL  SER  ALA  ASP  SER  THR  LEU  ARG  GLN  GLU  MET  ARG  GLN  GLN  GLU  GLY  THR
A G G A A A T A C C G T T T C T G C T G A T A G T A C T T T A C G T C A G G A A A T G C G C C A A C A A G A A G G A A C
           1450              1460              1470              1480              1490              1500

   TRP  TYR  ASN  SER  GLN  LEU  VAL  GLU  LEU  GLY  LYS  ILE  ARG  LEU  ASP  ARG  THR  GLY  PHE  PHE
T T G G T A T A A T T C A C A A T T A G T T G A G T T A G G A A A A A T T C G C T T A G A T C G T A C A G G T T T C T T
           1510              1520              1530              1540              1550              1560

   GLU  THR  VAL  GLU  ASN  ARG  ILE  ASP  PRO  ILE  ASN  GLY  SER  ASN  ASP  GLU  VAL  ASP  VAL  VAL
C G A A A C A G T T G A A A A C C G A A T T G A T C C T A T C A A T G G T A G C A A T G A T G A A G T G G A T G T C G T
           1570              1580              1590              1600              1610              1620

   TYR  LYS  VAL  LYS  GLU  ARG  ASN  THR  GLY  SER  ILE  ASN  PHE  GLY  ILE  GLY  TYR  GLY  THR  GLU
A T A T A A A G T C A A A G A A C G T A A C A C G G G T A G T A T C A A C T T T G G T A T T G G T T A C G G T A C A G A
           1630              1640              1650              1660              1670              1680

   SER  GLY  ILE  SER  TYR  GLN  ALA  SER  VAL  LYS  GLN  ASP  ASN  PHE  LEU  GLY  THR  GLY  ALA  ALA
G A G T G G T A T T A G T T A T C A A G C A A G T G T C A A A C A A G A T A A T T T C T T G G G A A C A G G G G C G G C
           1690              1700              1710              1720              1730              1740
```

FIG.1D.(CONTINUED)

VAL SER ILE ALA GLY THR LYS ASN ASP TYR GLY THR SER VAL ASN LEU GLY TYR THR GLU
AGTAAGTATAGCTGGTACGAAAAATGATTATGGTACGAGTGTCAATTTGGGTTATACCGA
1750 1760 1770 1780 1790 1800

PRO TYR PHE THR LYS ASP GLY VAL SER LEU GLY GLY ASN VAL PHE PHE GLU ASN TYR ASP
GCCCTATTTTACTAAAGATGGTGTAAGTCTTGGTGGAAATGTTTTCTTTGAAAACTACGA
1810 1820 1830 1840 1850 1860

ASN SER LYS SER ASP THR SER SER ASN TYR LYS ARG THR THR TYR GLY SER ASN VAL THR
TAACTCTAAAAGTGATACATCCTCTAACTATAAGCGTACGACTTATGGAAGTAATGTTAC
1870 1880 1890 1900 1910 1920

LEU GLY PHE PRO VAL ASN GLU ASN ASN SER TYR TYR VAL GLY LEU GLY HIS THR TYR ASN
TTTAGGTTTCCCTGTAAATGAAAATAACTCCTATTATGTAGGATTAGGCCATACCTATAA
1930 1940 1950 1960 1970 1980

LYS ILE SER ASN PHE ALA LEU GLU TYR ASN ARG ASN LEU TYR ILE GLN SER MET LYS PHE
TAAAATTAGTAACTTTGCTCTAGAATATAACCGTAATTTATATATTCAATCAATGAAATT
1990 2000 2010 2020 2030 2040

LYS GLY ASN GLY ILE LYS THR ASN ASP PHE ASP PHE SER PHE GLY TRP ASN TYR ASN SER
TAAAGGTAATGGCATTAAAACAAATGACTTTGATTTTTCTTTTGGTTGGAACTATAACAG
2050 2060 2070 2080 2090 2100

FIG.1D.(CONTINUED)

```
   LEU  ASN  ARG  GLY  TYR  PHE  PRO  THR  LYS  GLY  VAL  LYS  ALA  SER  LEU  GLY  GLY  ARG  VAL  THR
C C T T A A T A G A G G C T A T T T C C C A A C T A A A G G G G T T A A A G C A A G T C T T G G T G G A C G A G T T A C
             2110              2120              2130              2140              2150              2160

   ILE  PRO  GLY  SER  ASP  ASN  LYS  TYR  TYR  LYS  LEU  SER  ALA  ASP  VAL  GLN  GLY  PHE  TYR  PRO
A A T T C C A G G T T C T G A T A A C A A A T A C T A C A A A C T A A G T G C A G A T G T A C A G G G T T T C T A C C C
             2170              2180              2190              2200              2210              2220

   LEU  ASP  ARG  ASP  HIS  LEU  TRP  VAL  VAL  SER  ALA  LYS  ALA  SER  ALA  GLY  TYR  ALA  ASN  GLY
A T T A G A C A G A G A T C A C C T C T G G G T T G T A T C T G C A A A A G C A T C T G C A G G A T A T G C A A A T G G
             2230              2240              2250              2260              2270              2280

   PHE  GLY  ASN  LYS  ARG  LEU  PRO  PHE  TYR  GLN  THR  TYR  THR  ALA  GLY  GLY  ILE  GLY  SER  LEU
T T T T G G A A A C A A G C G T T T A C C G T T C T A T C A A A C T T A T A C A G C G G G T G G C A T T G G T T C A T T
             2290              2300              2310              2320              2330              2340

   ARG  GLY  PHE  ALA  TYR  GLY  SER  ILE  GLY  PRO  ASN  ALA  ILE  TYR  GLN  GLY  GLN  ASN  ASN  LYS
A C G C G G T T T T G C T T A T G G T A G C A T T G G G C C T A A C G C A A T T T A T C A A G G T C A A A A T A A T A A
             2350              2360              2370              2380              2390              2400

   PHE  ASN  LYS  ILE  SER  SER  ASP  VAL  ILE  GLY  GLY  ASN  ALA  ILE  ALA  THR  ALA  SER  ALA  GLU
A T T T A A T A A G A T A A G T T C T G A T G T G A T T G G T G G T A A T G C A A T C G C T A C A G C T A G C G C A G A
             2410              2420              2430              2440              2450              2460
```

FIG.1D.(CONTINUED)

```
   LEU  ILE  VAL  PRO  THR  PRO  PHE  VAL  SER  ASP  LYS  SER  GLN  ASN  THR  VAL  ARG  THR  SER  LEU
G T T A A T T G T G C C A A C T C C A T T T G T G A G T G A T A A G A G T C A A A A T A C A G T C C G A A C C T C C C T
            2470              2480              2490              2500              2510              2520

   PHE  VAL  ASP  ALA  ALA  SER  VAL  TRP  ASN  THR  LYS  TRP  LYS  SER  ASP  LYS  ASN  GLY  LEU  GLU
A T T T G T T G A T G C G G C A A G T G T T T G G A A T A C T A A A T G G A A A T C A G A T A A A A A T G G A T T A G A
            2530              2540              2550              2560              2570              2580

   SER  ASN  VAL  LEU  LYS  ASP  LEU  PRO  ASP  TYR  GLY  LYS  SER  SER  ARG  THR  ARG  ALA  SER  THR
G A G C A A T G T C T T G A A A G A C T T A C C C G A T T A T G G C A A A T C A A G C C G T A C T C G C G C C T C T A C
            2590              2600              2610              2620              2630              2640

   GLY  VAL  GLY  PHE  GLN  TRP  GLN  SER  PRO  SER  GLY  PRO  VAL  VAL  PHE  SER  TYR  ALA  LYS  PRO
A G G T G T C G G A T T C C A A T G G C A A T C T C C T A G T G G A C C A G T G G T A T T T T C T T A T G C T A A A C C
            2650              2660              2670              2680              2690              2700

   ILE  LYS  LYS  TYR  GLU  ASN  ASP  ASP  VAL  GLU  GLN  PHE  GLN  PHE  SER  ILE  GLY  GLY  SER  PHE
A A T T A A A A A A T A T G A A A A T G A T G A T G T C G A A C A G T T C C A A T T T A G T A T T G G G G G T T C T T T
            2710              2720              2730              2740              2750              2760

   ***  ***
C T A A T A A A T T G A A C T T T T T T C G T C A T C A G A A C T C A A A A A C A A C G T T C T C T G C C T A A T T T A
            2770              2780              2790              2800              2810              2820
```

FIG.1D.(CONTINUED)

```
ATTGGGCAGAGAAAATATTAAAACCATCATTTAATTAAGGATATTTATCAAATGAAAAAC
          2830      2840      2850      2860      2870      2880

ATCGCCAAAGTAACCGCACTTGCTTTAGGTATTGCACTTGCTTCAGGCTATGCTGCAGCT
          2890      2900      2910      2920      2930      2940

GAAGAAAAAATTGCTTTTATTAATGCAGGTTATA
          2950      2960      2970
```

FIG.1E.

JB-1042-9-4 DNA, PAK D15
IS THE SEQUENCE BEING TRANSLATED

```
AAAAGGCATTGAAAAAACAGGACAACTTTCCCTTTTAACCTTGAAAATATTAGGGAAATT
          10        20        30        40        50        60

ACTTACTGGCGATTTGTCATTAAATAATTTAAGTGGGCCAATTTCTATTGCAAAAGGTGC
          70        80        90       100       110       120

TGGTGCATCAGCAAATATTGGATTGGTGTATTTTTTAAGTTTTATGGCATTGATTAGTGT
         130       140       150       160       170       180

AAATTTAGGGATTATGAATTTATTTCCATTACCAGTATTAGATGGCGGTCATTTAGTTTT
         190       200       210       220       230       240

TTTAACAATGGAAGCTGTTAAAGGAAAACCTGTTTCTGAGCGGGTGCAAAGCATCTGTTA
         250       260       270       280       290       300

TCGAATTGGCGCAGCACTGTTATTAAGCTTAACGGTGTTTGCATTATTTAATGATTTTTT
         310       320       330       340       350       360
```

FIG.1E.(CONTINUED)

```
                                                    MET  LYS  LYS  LEU  LEU  ILE  ALA  SER  LEU  LEU  P
A C G T C T A T A A T T T A T A T A G G A T A C A A T C G A T G A A A A A A C T T C T A A T C G C A A G T T T A T T A T
              370                  380                  390                  400                  410                  420

HE  GLY  THR  THR  THR  THR  VAL  PHE  ALA  ALA  PRO  PHE  VAL  ALA  LYS  ASP  ILE  ARG  VAL  ASP  G
T C G G T A C G A C A A C G A C T G T G T T T G C C G C A C C T T T T G T G G C A A A A G A T A T T C G T G T G G A T G
              430                  440                  450                  460                  470                  480

LY  VAL  GLN  GLY  ASP  LEU  GLU  GLN  GLN  ILE  ARG  ALA  SER  LEU  PRO  VAL  ARG  ALA  GLY  GLN  A
G T G T T C A A G G T G A C T T A G A A C A A C A A A T C C G A G C A A G T T T A C C T G T T C G T G C T G G T C A G C
              490                  500                  510                  520                  530                  540

RG  VAL  THR  ASP  ASN  ASP  VAL  ALA  ASN  ILE  VAL  ARG  SER  LEU  PHE  VAL  SER  GLY  ARG  PHE  A
G T G T G A C T G A C A A T G A T G T G G C T A A T A T T G T C C G C T C T T T A T T C G T A A G T G G T C G A T T C G
              550                  560                  570                  580                  590                  600

SP  ASP  VAL  LYS  ALA  HIS  GLN  GLU  GLY  ASP  VAL  LEU  VAL  VAL  SER  VAL  VAL  ALA  LYS  SER  I
A T G A T G T G A A A G C G C A T C A A G A A G G C G A T G T G C T T G T T G T T A G C G T T G T G G C T A A A T C G A
              610                  620                  630                  640                  650                  660

LE  ILE  SER  ASP  VAL  LYS  ILE  LYS  GLY  ASN  SER  VAL  ILE  PRO  THR  GLU  ALA  LEU  LYS  GLN  A
T C A T T T C A G A T G T T A A A A T C A A A G G T A A C T C T G T T A T T C C C A C T G A A G C A C T T A A A C A A A
              670                  680                  690                  700                  710                  720
```

FIG.1E.(CONTINUED)

```
SN  LEU  ASP  ALA  ASN  GLY  PHE  LYS  VAL  GLY  ASP  VAL  LEU  ILE  ARG  GLU  LYS  LEU  ASN  GLU  P
A C T T A G A T G C T A A C G G G T T T A A A G T T G G C G A T G T T T T A A T T C G A G A A A A A T T A A A T G A A T
              730                  740                  750                  760                  770                  780

HE  ALA  LYS  SER  VAL  LYS  GLU  HIS  TYR  ALA  SER  VAL  GLY  ARG  TYR  ASN  ALA  THR  VAL  GLU  P
T T G C C A A A A G T G T A A A A G A G C A C T A T G C A A G T G T A G G T C G C T A T A A C G C A A C C G T T G A A C
              790                  800                  810                  820                  830                  840

RO  ILE  VAL  ASN  THR  LEU  PRO  ASN  ASN  ARG  ALA  GLU  ILE  LEU  ILE  GLN  ILE  ASN  GLU  ASP  A
C T A T T G T C A A T A C G C T G C C A A A T A A T C G T G C T G A A A T T T T A A T T C A A A T C A A T G A A G A T G
              850                  860                  870                  880                  890                  900

SP  LYS  ALA  LYS  LEU  ALA  SER  LEU  THR  PHE  LYS  GLY  ASN  GLU  SER  VAL  SER  SER  SER  THR  L
A T A A A G C A A A A T T G G C A T C A T T A A C T T T C A A G G G G A A C G A A T C T G T T A G T A G C A G T A C A T
              910                  920                  930                  940                  950                  960

EU  GLN  GLU  GLN  MET  GLU  LEU  GLN  PRO  ASP  SER  TRP  TRP  LYS  LEU  TRP  GLY  ASN  LYS  PHE  G
T A C A A G A A C A A A T G G A A T T A C A A C C T G A T T C T T G G T G G A A A T T A T G G G G A A A T A A A T T T G
              970                  980                  990                  1000                 1010                 1020

LU  GLY  ALA  GLN  PHE  GLU  LYS  ASP  LEU  GLN  ALA  ILE  ARG  ASP  TYR  TYR  LEU  ASN  ASN  GLY  T
A A G G T G C G C A A T T C G A G A A A G A T C T G C A G G C A A T T C G T G A T T A T T A T T T A A A T A A T G G C T
              1030                 1040                 1050                 1060                 1070                 1080

YR  ALA  LYS  ALA  GLN  ILE  THR  LYS  THR  ASP  VAL  GLN  LEU  ASN  ASP  GLU  LYS  THR  LYS  VAL  A
A T G C C A A A G C A C A A A T C A C T A A A A C G G A T G T T C A G C T A A A T G A T G A A A A A A A C A A A A A G T T A
              1090                 1100                 1110                 1120                 1130                 1140
```

FIG.1E.(CONTINUED)

```
SN  VAL  THR  ILE  ASP  VAL  ASN  GLU  GLY  LEU  GLN  TYR  ASP  LEU  ARG  SER  ALA  ARG  ILE  ILE  G
ATGTAACCATTGATGTAAATGAAGGTTTACAGTATGACCTTCGTAGTGCACGCATTATAG
          1150          1160          1170          1180          1190          1200

LY  ASN  LEU  GLY  GLY  MET  SER  ALA  GLU  LEU  GLU  PRO  LEU  LEU  SER  ALA  LEU  HIS  LEU  ASN  A
GTAATCTGGGAGGTATGTCTGCCGAGCTTGAACCTTTACTTTCAGCATTACATTTAAATG
          1210          1220          1230          1240          1250          1260

SP  THR  PHE  ARG  ARG  SER  ASP  ILE  ALA  ASP  VAL  GLU  ASN  ALA  ILE  LYS  ALA  LYS  LEU  GLY  G
ATACTTTCCGCCGTAGTGATATTGCAGATGTAGAAAATGCAATTAAAGCAAAACTTGGGG
          1270          1280          1290          1300          1310          1320

LU  ARG  GLY  TYR  GLY  ASN  THR  THR  VAL  ASN  SER  VAL  PRO  ASP  PHE  ASP  ASP  ALA  ASN  LYS  T
AACGAGGTTACGGTAACACAACAGTAAATTCTGTACCTGATTTTGACGATGCAAATAAAA
          1330          1340          1350          1360          1370          1380

HR  LEU  ALA  ILE  THR  PHE  VAL  VAL  ASP  ALA  GLY  ARG  ARG  LEU  THR  VAL  ARG  GLN  LEU  ARG  P
CATTAGCGATAACCTTTGTTGTTGATGCTGGACGACGTTTAACTGTTCGCCAACTTCGCT
          1390          1400          1410          1420          1430          1440

HE  GLU  GLY  ASN  THR  VAL  SER  ALA  ASP  SER  THR  LEU  ARG  GLN  GLU  MET  ARG  GLN  GLN  GLU  G
TTGAAGGAAATACCGTTTCTGCTGATAGTACTTTACGTCAGGAAATGCGACAACAAGAAG
          1450          1460          1470          1480          1490          1500
```

FIG.1E.(CONTINUED)

```
LY  THR  TRP  TYR  ASN  SER  GLN  LEU  VAL  GLU  LEU  GLY  LYS  ILE  ARG  LEU  ASP  ARG  THR  GLY  P
GAACTTGGTATAATTCACAATTAGTTGAGTTAGGAAAAATTCGCTTAGATCGTACAGGTT
            1510              1520              1530              1540              1550              1560

HE  PHE  GLU  THR  VAL  GLU  ASN  ARG  ILE  ASP  PRO  ILE  ASN  GLY  SER  ASN  ASP  GLU  VAL  ASP  V
TCTTCGAAACAGTTGAAAACCGAATTGATCCTATCAATGGTAGCAATGATGAAGTGGATG
            1570              1580              1590              1600              1610              1620

AL  VAL  TYR  LYS  VAL  LYS  GLU  ARG  ASN  THR  GLY  SER  ILE  ASN  PHE  GLY  ILE  GLY  TYR  GLY  T
TCGTATATAAAGTCAAAGAACGTAACACGGGTAGTATCAACTTTGGTATTGGTTACGGTA
            1630              1640              1650              1660              1670              1680

HR  GLU  SER  GLY  ILE  SER  TYR  GLN  THR  SER  ILE  LYS  GLN  ASP  ASN  PHE  LEU  GLY  THR  GLY  A
CAGAGAGTGGTATCAGTTATCAAACAAGTATTAAACAAGATAATTTCTTGGGAACAGGGG
            1690              1700              1710              1720              1730              1740

LA  ALA  VAL  SER  ILE  ALA  GLY  THR  LYS  ASN  ASP  TYR  GLY  THR  SER  VAL  ASN  LEU  GLY  TYR  T
CGGCAGTAAGTATAGCTGGTACGAAAAATGATTATGGTACGAGTGTCAATTTGGGTTATA
            1750              1760              1770              1780              1790              1800

HR  GLU  PRO  TYR  PHE  THR  LYS  ASP  GLY  VAL  SER  LEU  GLY  GLY  ASN  ILE  PHE  PHE  GLU  ASN  T
CCGAACCCTATTTTACTAAAGATGGTGTAAGTCTTGGTGGAAATATTTTCTTTGAAAACT
            1810              1820              1830              1840              1850              1860
```

FIG.1E.(CONTINUED)

YR ASP ASN SER LYS SER ASP THR SER SER ASN TYR LYS ARG THR THR TYR GLY SER ASN V
A C G A T A A C T C T A A A A G T G A T A C A T C C T C T A A C T A T A A G C G T A C G A C T T A T G G A A G T A A T G
1870 1880 1890 1900 1910 1920

AL THR LEU GLY PHE PRO VAL ASN GLU ASN ASN SER TYR TYR VAL GLY LEU GLY HIS THR T
T T A C T T T A G G T T T C C C T G T A A A T G A A A A T A A C T C C T A T T A T G T A G G A T T A G G C C A T A C C T
1930 1940 1950 1960 1970 1980

YR ASN LYS ILE SER ASN PHE ALA LEU GLU TYR ASN ARG ASN LEU TYR ILE GLN SER MET L
A T A A T A A A A T T A G T A A C T T T G C T C T A G A A T A T A A C C G T A A T T T A T A T A T T C A A T C A A T G A
1990 2000 2010 2020 2030 2040

YS PHE LYS GLY ASN GLY ILE LYS THR ASN ASP PHE ASP PHE SER PHE GLY TRP ASN TYR A
A A T T T A A A G G T A A T G G C A T T A A A A C A A A T G A C T T T G A T T T T T C T T T T G G T T G G A A C T A T A
2050 2060 2070 2080 2090 2100

SN SER LEU ASN ARG GLY TYR PHE PRO THR LYS GLY VAL LYS ALA SER LEU GLY GLY ARG V
A C A G C C T T A A T A G A G G C T A T T T C C C A A C T A A A G G G G T T A A A G C A A G T C T T G G T G G A C G A G
2110 2120 2130 2140 2150 2160

AL THR ILE PRO GLY SER ASP ASN LYS TYR TYR LYS LEU SER ALA ASP VAL GLN GLY PHE T
T T A C T A T T C C A G G T T C T G A T A A C A A A T A C T A C A A A C T A A G T G C A G A T G T A C A G G G T T T C T
2170 2180 2190 2200 2210 2220

YR PRO LEU ASP ARG ASP HIS ARG TRP VAL VAL SER ALA LYS ALA SER ALA GLY TYR ALA A
A C C C A T T A G A C A G A G A T C A C C G C T G G G T T G T A T C T G C A A A A G C A T C T G C A G G A T A T G C A A
2230 2240 2250 2260 2270 2280

FIG.1E.(CONTINUED)

```
SN  GLY  PHE  GLY  ASN  LYS  ARG  LEU  PRO  PHE  TYR  GLN  THR  TYR  THR  ALA  GLY  GLY  ILE  GLY  S
ATGGTTTTGGAAACAAGCGTTTACCGTTCTATCAAACTTATACAGCGGGTGGCATTGGTT
              2290              2300              2310              2320              2330              2340

ER  LEU  ARG  GLY  PHE  ALA  TYR  GLY  SER  ILE  GLY  PRO  ASN  ALA  ILE  TYR  ALA  GLU  HIS  GLY  A
CATTACGCGGTTTTGCTTATGGTAGTATTGGGCCTAATGCAATTTATGCCGAACATGGTA
              2350              2360              2370              2380              2390              2400

SN  GLY  THR  PHE  ASN  LYS  ILE  SER  SER  ASP  VAL  ILE  GLY  GLY  ASN  ALA  ILE  THR  THR  ALA  S
ATGGTACTTTTAATAAGATAAGTTCTGATGTGATTGGTGGTAATGCAATCACAACTGCGA
              2410              2420              2430              2440              2450              2460

ER  ALA  GLU  LEU  ILE  VAL  PRO  THR  PRO  PHE  VAL  SER  ASP  LYS  SER  GLN  ASN  THR  VAL  ARG  T
GTGCAGAACTTATTGTACCAACTCCATTTGTGAGTGATAAAAGCCAAAATACAGTCCGAA
              2470              2480              2490              2500              2510              2520

HR  SER  LEU  PHE  VAL  ASP  ALA  ALA  SER  VAL  TRP  ASN  THR  LYS  TRP  LYS  SER  ASP  LYS  ASN  G
CCTCCCTATTTGTTGATGCGGCAAGTGTTTGGAATACTAAATGGAAATCAGATAAAAATG
              2530              2540              2550              2560              2570              2580

LY  LEU  GLU  SER  LYS  VAL  LEU  LYS  ASP  LEU  PRO  ASP  TYR  GLY  LYS  SER  SER  ARG  ILE  ARG  A
GATTAGAGAGCAAGGTCTTGAAAGACTTACCTGATTATGGCAAATCAAGCCGTATTCGCG
              2590              2600              2610              2620              2630              2640
```

FIG.1E.(CONTINUED)

```
LA  SER  THR  GLY  VAL  GLY  PHE  GLN  TRP  GLN  SER  PRO  ILE  GLY  PRO  LEU  VAL  PHE  SER  TYR  A
CCTCTACAGGTGTCGGATTCCAATGGCAATCTCCTATTGGACCATTGGTATTTTCTTATG
            2650              2660              2670              2680              2690              2700

LA  LYS  PRO  ILE  LYS  LYS  TYR  GLU  ASN  ASP  ASP  VAL  GLU  GLN  PHE  GLN  PHE  SER  ILE  GLY  G
CTAAACCAATTAAAAAAATATGAAAATGATGATGTCGAACAGTTCCAATTTAGTATTGGGG
            2710              2720              2730              2740              2750              2760

LY  SER  PHE  ***  ***
GCTCTTTCTAATAAATTGAACTTTTTTCGTCATCAGAACTCAAAAACGACGTTCTCTGCC
            2770              2780              2790              2800              2810              2820

TAATTGAATTGGGCAGAGAAAATATTAAACCCATCATTTAATTAAGGATATTTATCAAAT
            2830              2840              2850              2860              2870              2880

GAAAAACATCGCAAAAGTAACCGCACTTGCTTTAGGTTTTGCACTTGCTTCAGGCTATGC
            2890              2900              2910              2920              2930              2940

TTCCGCTGAAGAAAAAATTGCTTTCATTAATGCAGGTTATATTTTTCAA
            2950              2960              2970              2980
```

FIG.1F.

```
1. cad15          (1-2949)
3. minnad15       (1-2953)
2. eagand15       (1-2984)
4. pakd15         (1-2989)
5. sb33d15        (1-2974)

cad15       1

minnad15    1

eagand15    1                ACAGGACAgCTTTCCCTTTTAACCTTGAAAATATTAGGGAAATTA
                             |||||||||  |||||||||||||||||||||||||||||||||||
pakd15      1  aaaaGGCATTGAAAAAACAGGACAaCTTTCCCTTTTAACCTTGAAAATATTAGGGAAATTA
                 ||||||||||||||||||||  |||||||||||||||||||||||||||||||||||
sb33d15     1        GGCATTGAAAAAACAGGACAgCTTTCCCTTTTAACCTTGAAAATATTAGGGAAATTA

consensus           aaaaggcattgaaaaaacaggacagctttcccttttaaccttgaaaatattagggaaatta

cad15       1
```

FIG.1F.(CONTINUED)

```
minnad15      6 CTTaCTGGCGATTTGTCATTAAATAATTTAAGTGGGCCAATTTCTATTGCAAAAGGTGCTG
                |||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15     46 CTTcCTGGCGATTTGTCATTAAATAATTTAAGTGGGCCAATTTCTATTGCAAAAGGTGCTG
                |||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15       62 CTTACTGGCGATTTGTCATTAAATAATTTAAGTGGGCCAATTTCTATTGCAAAAGGTGCTG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15      58 CTTACTGGCGATTTGTCATTAAATAATTTAAGTGGGCCAATTTCTATTGCAAAAGGTGCTG

consensus        cttactggcgatttgtcattaaataatttaagtgggccaatttctattgcaaaaggtgctg


cad15         1

minnad15     67 GCaCATCAGCAAATATTGGATTGGTGTATTTTTTAAGTTTTATGGCACTGATTAGTGTAAA
                ||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15    107 GCcCATCAGCAAATATTGGATTGGTGTATTTTTTAAGTTTTATGGCACTGATTAGTGTAAA
                |   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15      123 GtGCATCAGCAAATATTGGATTGGTGTATTTTTTAAGTTTTATGGCATTGATTAGTGTAAA
                |  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15     119 GcGCATCAGCAAATATTGGATTGGTGTATTTTTTAAGTTTTATGGCATTGATTAGTGTAAA

consensus        gcgcatcagcaaatattggattggtgtatttttaagttttatggca-tgattagtgtaaa
```

FIG.1F.(CONTINUED)

```
cad15       1                            gATTAC
                                          |||||
minnad15  128  TTTAGGATTATGAATTTATTTCCATTACCAGTATTAGATGGCGGTCATTTAGTTTTTTA
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15  168  TTTAGGATTATGAATTTATTTCCATTACCAGTATTAGATGGCGGTCATTTAGTTTTTTA
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15    184  TTTAGGATTATGAATTTATTTCCATTACCAGTATTAGATGGCGGTCATTTAGTTTTTTA
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15   180  TTTAGGATTATGAATTTATTTCCATTACCAGTATTAGATGGCGGTCATTTAGTTTTTTA

consensus      tttagggattatgaatttatttccATTACcagtattagatggcggtcatttagtttttta

cad15       7
minnad15  189  ACAATGGAAGCTGTTAAAGGAAAACCTGTTTCTGAGCGGGTGCAAAGCATCTGTTATCGAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15  229  ACAATGGAAGCTGTTAAAGGAAAACCTGTTTCTGAGCGGGTGCAAAGCATCTGTTATCGAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15    245  ACAATGGAAGCTGTTAAAGGAAAACCTGTTTCTGAGCGGGTGCAAAGCATCTGTTATCGAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15   241  ACAATGGAAGCTGTTAAAGGAAAACCTGTTTCTGAGCGGGTGCAAAGCATCTGTTATCGAA
```

FIG.1F.(CONTINUED)

```
consensus        acaatggaagctgttaaaggaaaacctgtttctgagcgggtgcaaagcatctgttatcgaa

    cad15      7                                gccAAGCTTAACGGTGTTTGCATTATTTAATGATTTTTTACGTCT
                                                ||||||||||||||||||||||||||||||||||||||||||||
  minnad15   250 TTGGCGCAGCACTGTTATTAAGCTTAACGGTGTTTGCATTATTTAATGATTTTTTACGTCT
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  eagand15   290 TTGGCGCAGCACTGTTATTAAGCTTAACGGTGTTTGCATTATTTAATGATTTTTTACGTCT
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    pakd15   306 TTGGCGCAGCACTGTTATTAAGCTTAACGGTGTTTGCATTATTTAATGATTTTTTACGTCT
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    sb33d15  302 TTGGCGCAGCACTGTTATTAAGCTTAACGGTGTTTGCATTATTTAATGATTTTTTACGTCT

consensus        ttggcgcagcactgttattAAGCTTAACGGTGTTTGCATTATTTAATGATTTTTTACGTCT

    cad15     52 ATAATTTATATAGGATACAATCGATGAAAAAACTTCTAATCGCAAGTTTATTATTCGGTAC
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  minnad15   311 ATAATTTATATAGGATACAATCGATGAAAAAACTTCTAATCGCAAGTTTATTATTCGGTAC
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  eagand15   351 ATAATTTATATAGGATACAATCGATGAAAAAACTTCTAATCGCAAGTTTATTATTCGGTAC
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
    pakd15   367 ATAATTTATATAGGATACAATCGATGAAAAAACTTCTAATCGCAAGTTTATTATTCGGTgC
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
```

FIG.1F.(CONTINUED)

```
sb33d15   363 ATAATTTATATAGGATACAATCGATGAAAAAACTTCTAATCGCAAGTTTATTATTCGGTaC

consensus     ATAATTTATATAGGATACAATCGATGAAAAAACTTCTAATCGCAAGTTTATTATTCGGTaC


cad15     113 GACAACGACTGTGTTTGCCGCACCTTTTGTGGCAAAAGATATTCGTGTGGATGGTGTTCAA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15  372 GACAACGACTGTGTTTGCCGCACCTTTTGTGGCAAAAGATATTCGTGTGGATGGTGTTCAA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15  412 GACAACGACTGTGTTTGCCGCACCTTTTGTGGCAAAAGATATTCGTGTGGATGGTGTTCAA
              |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
pakd15    428 GACAACGACTGTGTTTGCCGCACCTTTTGTGcCAAAAGATATTCGTGTGGATGGTGTTCAA
              |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
sb33d15   424 GACAACGACTGTGTTTGCCGCACCTTTTGTGgCAAAAGATATTCGTGTGGATGGTGTTCAA

consensus     GACAACGACTGTGTTTGCCGCACCTTTTGTGgCAAAAGATATTCGTGTGGATGGTGTTCAA


cad15     174 GGTGACTTAGAACAACAAATCCGAGCAAGTTTACCTGTTCGTGCCGGTCAGCGTGTGACTG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15  433 GGTGACTTAGAACAACAAATCCGAGCAAGTTTACCTGTTCGTGCCGGTCAGCGTGTGACTG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15  473 GGTGACTTAGAACAACAAATCCGAGCAAGTTTACCTGTTCGTGCCGGTCAGCGTGTGACTG
              |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
```

FIG.1F.(CONTINUED)

```
pakd15      489 GGTGACTTAGAACAACAAATCCGAGCAAGTTTACCTGTTCGTGCtGGTCAGCGTGTGACTG
                ||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
sb33d15     485 GGTGACTTAGAACAACAAATCCGAGCAAGTTTACCTGTTCGTGCcGGTCAGCGTGTGACTG

consensus       GGTGACTTAGAACAACAAATCCGAGCAAGTTTACCTGTTCGTGCcGGTCAGCGTGTGACTG


cad15       235 ACAATGATGTGGCTAATATTGTCCGCTCTTTATTCGTAAGTGGTCGATTCGATGATGTGAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15    494 ACAATGATGTGGCTAATATTGTCCGCTCTTTATTCGTAAGTGGTCGATTCGATGATGTGAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15    534 ACAATGATGTGGCTAATATTGTCCGCTCTTTATTCGTAAGTGGTCGATTCGATGATGTGAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15      550 ACAATGATGTGGCTAATATTGTCCGCTCTTTATTCGTAAGTGGTCGATTCGATGATGTGAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15     546 ACAATGATGTGGCTAATATTGTCCGCTCTTTATTCGTAAGTGGTCGATTCGATGATGTGAA

consensus       ACAATGATGTGGCTAATATTGTCCGCTCTTTATTCGTAAGTGGTCGATTCGATGATGTGAA


cad15       296 AGCGCATCAAGAAGGCGATGTGCTTGTTGTTAGCGTTGTGGCTAAATCGATCATTTCAGAT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15    555 AGCGCATCAAGAAGGCGATGTGCTTGTTGTTAGCGTTGTGGCTAAATCGATCATTTCAGAT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

FIG.1F.(CONTINUED)

```
eagand15   595 AGCGCATCAAGAAGGCGATGTGCTTGTTGTTAGCGTTGTGGCTAAATCGATCATTTCAGAT
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15     611 AGCGCATCAAGAAGGCGATGTGCTTGTTGTTAGCGTTGTGGCTAAATCGATCATTTCAGAT
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15    607 AGCGCATCAAGAAGGCGATGTGCTTGTTGTTAGCGTTGTGGCTAAATCGATCATTTCAGAT

consensus      AGCGCATCAAGAAGGCGATGTGCTTGTTGTTAGCGTTGTGGCTAAATCGATCATTTCAGAT


cad15      357 GTTAAAATCAAAGGTAACTCTGTTATTCCCACTGAAGCACTTAAACAAAACTTAGATGCTA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15   616 GTTAAAATCAAAGGTAACTCTGTTATTCCCACTGAAGCACTTAAACAAAACTTAGATGCTA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15   656 GTTAAAATCAAAGGTAACTCTGTTATTCCCACTGAAGCACTTAAACAAAACTTAGATGCTA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15     672 GTTAAAATCAAAGGTAACTCTGTTATTCCCACTGAAGCACTTAAACAAAACTTAGATGCTA
               |||||||||||||||||||| ||||||| |||||||||| ||||||||||||||||||||
sb33d15    668 GTTAAAATCAAAGGTAACTCTaTTATTCCacCTGAAGCACTaAAACAAAACTTAGATGCTA

consensus      GTTAAAATCAAAGGTAACTCTgTTATTCCcaCTGAAGCACTtAAACAAAACTTAGATGCTA


cad15      418 ACGGGTTTAAAGTTGGCGATGTTTTAATTCGAGAAAAATTAAATGAATTTGCCAAAAGTGT
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

FIG.1F.(CONTINUED)

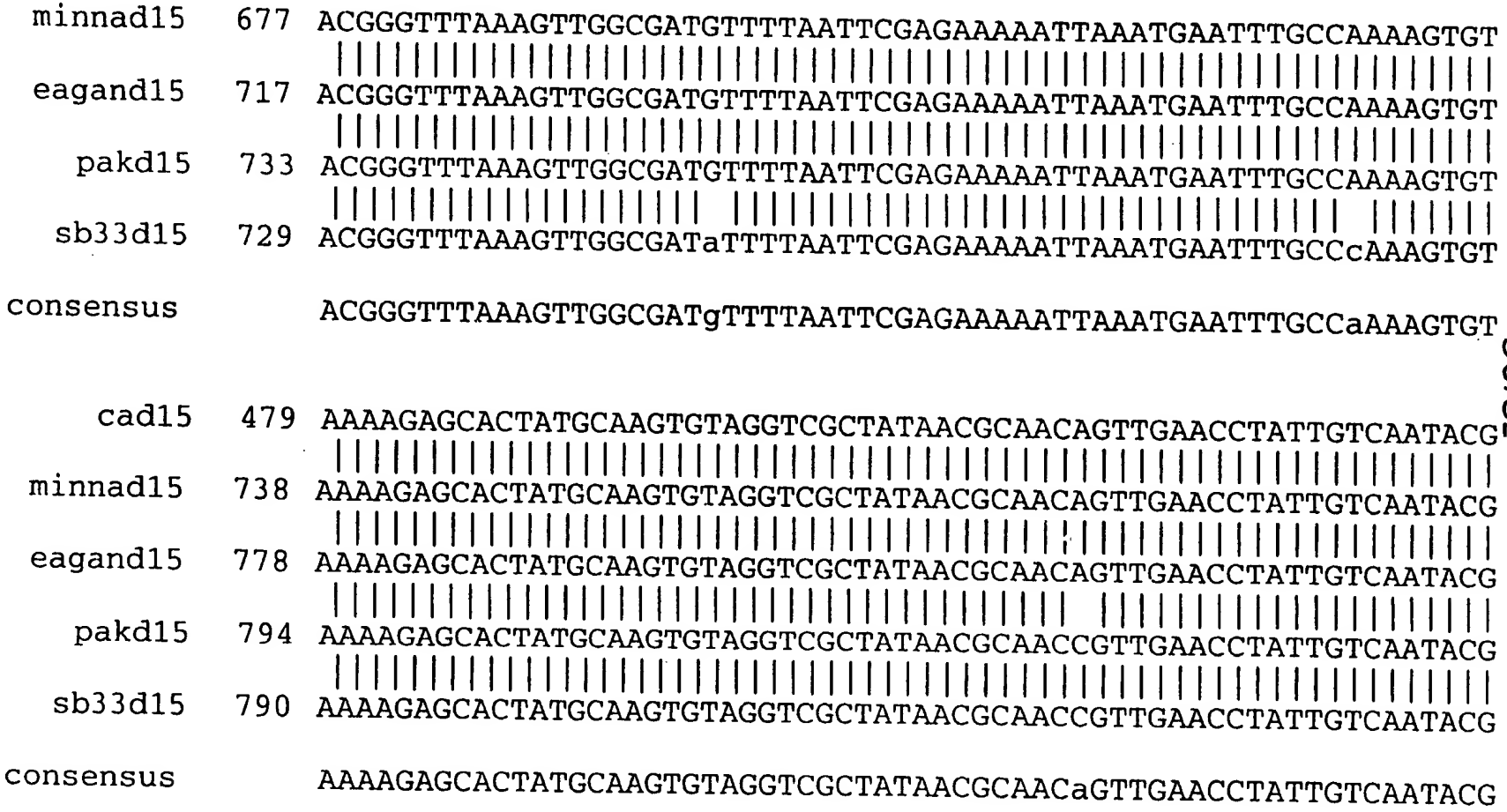

FIG.1F.(CONTINUED)

```
cad15      540 CTACCAAATAATCGCGCTGAAATTTTAATTCAAATCAATGAAGATGATAAAGCAAAATTGG
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15   799 CTACCAAATAATCGCGCTGAAATTTTAATTCAAATCAATGAAGATGATAAAGCAAAATTGG
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15   839 CTACCAAATAATCGCGCTGAAATTTTAATTCAAATCAATGAAGATGATAAAGCAAAATTGG
               || |||||||||||  ||||||||||||||||||||||||||||||||||||||||||||
pakd15     855 CTgCCAAATAATCGtGCTGAAATTTTAATTCAAATCAATGAAGATGATAAAGCAAAATTGG
               || |||||||||||  ||||||||||||||||||||||||||||||||||||  |||||||
sb33d15    851 CTaCCAAATAATCGcGCTGAAATTTTAATTCAAATCAATGAAGATGATAAAGCcAAATTGG
consensus      CTaCCAAATAATCGcGCTGAAATTTTAATTCAAATCAATGAAGATGATAAAGCaAAATTGG

cad15      601 CATCATTAACTTTCAAGGGGAACGAATCTGTTAGTAGCAGTACATTACAAGAACAAATGGA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15   860 CATCATTAACTTTCAAGGGGAACGAATCTGTTAGTAGCAGTACATTACAAGAACAAATGGA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15   900 CATCATTAACTTTCAAGGGGAACGAATCTGTTAGTAGCAGTACATTACAAGAACAAATGGA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15     916 CATCATTAACTTTCAAGGGGAACGAATCTGTTAGTAGCAGTACATTACAAGAACAAATGGA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15    912 CATCATTAACTTTCAAGGGGAACGAATCTGTTAGTAGCAGTACATTACAAGAACAAATGGA
consensus      CATCATTAACTTTCAAGGGGAACGAATCTGTTAGTAGCAGTACATTACAAGAACAAATGGA
```

FIG.1F.(CONTINUED)

```
cad15     662 ATTACAACCTGATTCTTGGTGGAAATTATGGGGAAATAAATTTGAAGGTGCGCAATTCGAG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15  921 ATTACAACCTGATTCTTGGTGGAAATTATGGGGAAATAAATTTGAAGGTGCGCAATTCGAG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15  961 ATTACAACCTGATTCTTGGTGGAAATTATGGGGAAATAAATTTGAAGGTGCGCAATTCGAG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15    977 ATTACAACCTGATTCTTGGTGGAAATTATGGGGAAATAAATTTGAAGGTGCGCAATTCGAG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15   973 ATTACAACCTGATTCTTGGTGGAAATTATGGGGAAATAAATTTGAAGGTGCGCAATTCGAG

consensus     ATTACAACCTGATTCTTGGTGGAAATTATGGGGAAATAAATTTGAAGGTGCGCAATTCGAG


cad15     723 AAAGATTTGCAGTCAATTCGTGATTATTATTTAAATAATGGCTATGCCAAAGCACAAATTA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15  982 AAAGATTTGCAGTCAATTCGTGATTATTATTTAAATAATGGCTATGCCAAAGCACAAATTA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15 1022 AAAGATTTGCAGTCAATTCGTGATTATTATTTAAATAATGGCTATGCCAAAGCACAAATTA
              |||||| |||||  |||||||||||||||||||||||||||||||||||||||||||||  |
pakd15   1038 AAAGATcTGCAGGCAATTCGTGATTATTATTTAAATAATGGCTATGCCAAAGCACAAATCA
              |||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15  1034 AAAGATtTGCAGGCAATTCGTGATTATTATTTAAATAATGGCTATGCCAAAGCACAAATCA

consensus     AAAGATtTGCAGtCAATTCGTGATTATTATTTAAATAATGGCTATGCCAAAGCACAAATtA
```

FIG.1F.(CONTINUED)

```
cad15     784 CTAAAACGGATGTTCAGCTAAATGATGAAAAAACAAAAGTTAATGTAACCATTGATGTAAA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15 1043 CTAAAACGGATGTTCAGCTAAATGATGAAAAAACAAAAGTTAATGTAACCATTGATGTAAA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15 1083 CTAAAACGGATGTTCAGCTAAATGATGAAAAAACAAAAGTTAATGTAACCATTGATGTAAA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15    1099 CTAAAACGGATGTTCAGCTAAATGATGAAAAAACAAAAGTTAATGTAACCATTGATGTAAA
              ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15   1095 CTAAAgCGGATGTTCAGCTAAATGATGAAAAAACAAAAGTTAATGTAACCATTGATGTAAA
consensus      CTAAAaCGGATGTTCAGCTAAATGATGAAAAAACAAAAGTTAATGTAACCATTGATGTAAA

cad15     845 TGAAGGTTTACAGTATGACCTTCGTAGTGCACGCATTATAGGTAATCTGGGAGGTATGTCT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15 1104 TGAAGGTTTACAGTATGACCTTCGTAGTGCACGCATTATAGGTAATCTGGGAGGTATGTCT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15 1144 TGAAGGTTTACAGTATGACCTTCGTAGTGCACGCATTATAGGTAATCTGGGAGGTATGTCT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15    1160 TGAAGGTTTACAGTATGACCTTCGTAGTGCACGCATTATAGGTAATCTGGGAGGTATGTCT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15   1156 TGAAGGTTTACAGTATGACCTTCGTAGTGCACGCATTATAGGTAATCTGGGAGGTATGTCT
consensus      TGAAGGTTTACAGTATGACCTTCGTAGTGCACGCATTATAGGTAATCTGGGAGGTATGTCT
```

FIG.1F.(CONTINUED)

```
     cad15   906 GCCGAGCTTGAACCTTTACTTTCAGCATTACATTTAAATGATACTTTCCGCCGTAGTGATA
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  minnad15  1165 GCCGAGCTTGAACCTTTACTTTCAGCATTACATTTAAATGATACTTTCCGCCGTAGTGATA
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  eagand15  1205 GCCGAGCTTGAACCTTTACTTTCAGCATTACATTTAAATGATACTTTCCGCCGTAGTGATA
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    pakd15  1221 GCCGAGCTTGAACCTTTACTTTCAGCATTACATTTAAATGATACTTTCCGCCGTAGTGATA
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   sb33d15  1217 GCCGAGCTTGAACCTTTACTTTCAGCATTACATTTAAATGATACTTTCCGCCGTAGTGATA

consensus        GCCGAGCTTGAACCTTTACTTTCAGCATTACATTTAAATGATACTTTCCGCCGTAGTGATA

     cad15   967 TTGCAGATGTAGAAAATGCAATTAAAGCAAAACTTGGAGAACGCGGTTACGGTAGCGCAAC
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  minnad15  1226 TTGCAGATGTAGAAAATGCAATTAAAGCAAAACTTGGAGAACGCGGTTACGGTAGCGCAAC
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  eagand15  1266 TTGCAGATGTAGAAAATGCAATTAAAGCAAAACTTGGAGAACGCGGTTACGGTAGCGCAAC
                 |||||||||||||||||||||||||||||||||| ||||| |||||||||| | ||||
    pakd15  1282 TTGCAGATGTAGAAAATGCAATTAAAGCAAAACTTGGGGAACGAGGTTACGGTAACACAAC
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   sb33d15  1278 TTGCAGATGTAGAAAATGCAATTAAAGCAAAACTTGGGGAACGAGGTTACGGTAACACAAC

consensus        TTGCAGATGTAGAAAATGCAATTAAAGCAAAACTTGGaGAACGcGGTTACGGTAgCgCAAC
```

FIG.1F.(CONTINUED)

```
cad15      1028 GGTAAATTCAGTACCTGATTTTGATGATGCAAATAAAACATTAGCGATAACCCTTGTTGTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15   1287 GGTAAATTCAGTACCTGATTTTGATGATGCAAATAAAACATTAGCGATAACCCTTGTTGTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15   1327 GGTAAATTCAGTACCTGATTTTGATGATGCAAATAAAACATTAGCGATAACCCTTGTTGTT
                 |||||||| ||||||||||||||| |||||||||||||||||||||||| ||||||||
pakd15     1343 AGTAAATTCTGTACCTGATTTTGACGATGCAAATAAAACATTAGCGATAACCTTTGTTGTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15    1339 AGTAAATTCTGTACCTGATTTTGACGATGCAAATAAAACATTAGCGATAACCTTTGTTGTT

consensus       gGTAAATTCaGTACCTGATTTTGAtGATGCAAATAAAACATTAGCGATAACCcTTGTTGTT


cad15      1089 GATGCTGGACGACGTTTAACTGTTCGCCAACTTCGCTTTGAAGGAAATACCGTTTCTGCTG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15   1348 GATGCTGGACGACGTTTAACTGTTCGCCAACTTCGCTTTGAAGGAAATACCGTTTCTGCTG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15   1388 GATGCTGGACGACGTTTAACTGTTCGCCAACTTCGCTTTGAAGGAAATACCGTTTCTGCTG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15     1404 GATGCTGGACGACGTTTAACTGTTCGCCAACTTCGCTTTGAAGGAAATACCGTTTCTGCTG
                |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
sb33d15    1400 GATGCTGGACGACGTTTAACTGTTCaCCAACTTCGCTTTGAAGGAAATACCGTTTCTGCTG

consensus       GATGCTGGACGACGTTTAACTGTTCgCCAACTTCGCTTTGAAGGAAATACCGTTTCTGCTG
```

FIG.1F.(CONTINUED)

```
cad15     1150  ATAGCACTTTACGTCAGGAAATGCGCCAACAAGAAGGAACTTGGTATAATTCACAATTAGT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15  1409  ATAGCACTTTACGTCAGGAAATGCGCCAACAAGAAGGAACTTGGTATAATTCACAATTAGT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15  1449  ATAGCACTTTACGTCAGGAAATGCGCCAACAAGAAGGAACTTGGTATAATTCACAATTAGT
                ||||  ||||||||||||||||||||  ||||||||||||||||||||||||||||||||
pakd15    1465  ATAGTACTTTACGTCAGGAAATGCGaCAACAAGAAGGAACTTGGTATAATTCACAATTAGT
                ||||||||||||||||||||||||||  ||||||||||||||||||||||||||||||||
sb33d15   1461  ATAGTACTTTACGTCAGGAAATGCGcCAACAAGAAGGAACTTGGTATAATTCACAATTAGT

consensus       ATAGcACTTTACGTCAGGAAATGCGcCAACAAGAAGGAACTTGGTATAATTCACAATTAGT

                                                                        56/82

cad15     1211  TGAGTTAGGAAAAATTCGCTTAGATCGTACAGGTTTCTTCGAAACAGTCGAAAACCGAATT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15  1470  TGAGTTAGGAAAAATTCGCTTAGATCGTACAGGTTTCTTCGAAACAGTCGAAAACCGAATT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15  1510  TGAGTTAGGAAAAATTCGCTTAGATCGTACAGGTTTCTTCGAAACAGTCGAAAACCGAATT
                ||||||||||||||||||||||||||||||||||||||||||||||  ||||||||||||
pakd15    1526  TGAGTTAGGAAAAATTCGCTTAGATCGTACAGGTTTCTTCGAAACAGTTGAAAACCGAATT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15   1522  TGAGTTAGGAAAAATTCGCTTAGATCGTACAGGTTTCTTCGAAACAGTTGAAAACCGAATT

consensus       TGAGTTAGGAAAAATTCGCTTAGATCGTACAGGTTTCTTCGAAACAGTcGAAAACCGAATT
```

FIG.1F.(CONTINUED)

```
cad15     1272  GATCCTATCAATGGTAGTAATGATGAAGTGGATGTCGTATATAAAGTCAAAGAACGTAACA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15  1531  GATCCTATCAATGGTAGTAATGATGAAGTGGATGTCGTATATAAAGTCAAAGAACGTAACA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15  1571  GATCCTATCAATGGTAGTAATGATGAAGTGGATGTCGTATATAAAGTCAAAGAACGTAACA
                |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
pakd15    1587  GATCCTATCAATGGTAGCAATGATGAAGTGGATGTCGTATATAAAGTCAAAGAACGTAACA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15   1583  GATCCTATCAATGGTAGCAATGATGAAGTGGATGTCGTATATAAAGTCAAAGAACGTAACA

consensus       GATCCTATCAATGGTAGtAATGATGAAGTGGATGTCGTATATAAAGTCAAAGAACGTAACA


cad15     1333  CGGGTAGTATCAACTTTGGTATTGGTTACGGTACAGAGAGTGGTATTAGTTATCAAGCAAG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15  1592  CGGGTAGTATCAACTTTGGTATTGGTTACGGTACAGAGAGTGGTATTAGTTATCAAGCAAG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15  1632  CGGGTAGTATCAACTTTGGTATTGGTTACGGTACAGAGAGTGGTATTAGTTATCAAGCAAG
                |||||||||||||||||||||||||||||||||||||||||||| |||||||| ||||
pakd15    1648  CGGGTAGTATCAACTTTGGTATTGGTTACGGTACAGAGAGTGGTATcAGTTATCAAaCAAG
                |||||||||||||||||||||||||||||||||||||||||||| |||||||| ||||
sb33d15   1644  CGGGTAGTATCAACTTTGGTATTGGTTACGGTACAGAGAGTGGTATtAGTTATCAAgCAAG

consensus       CGGGTAGTATCAACTTTGGTATTGGTTACGGTACAGAGAGTGGTATtAGTTATCAAgCAAG
```

FIG.1F.(CONTINUED)

```
     cad15 1394  TGTTAAACAAGATAATTTCTTGGGAACAGGGGCGGCAGTAAGTATAGCTGGTACGAAAAAT
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   minnad15 1653 TGTTAAACAAGATAATTTCTTGGGAACAGGGGCGGCAGTAAGTATAGCTGGTACGAAAAAT
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   eagand15 1693 TGTTAAACAAGATAATTTCTTGGGAACAGGGGCGGCAGTAAGTATAGCTGGTACGAAAAAT
                 | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     pakd15 1709 TaTTAAACAAGATAATTTCTTGGGAACAGGGGCGGCAGTAAGTATAGCTGGTACGAAAAAT
                 | | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    sb33d15 1705 TgTcAAACAAGATAATTTCTTGGGAACAGGGGCGGCAGTAAGTATAGCTGGTACGAAAAAT

consensus        TgTtAAACAAGATAATTTCTTGGGAACAGGGGCGGCAGTAAGTATAGCTGGTACGAAAAAT

     cad15 1455  GATTATGGTACGAGTGTCAATTTGGGTTATACCGAGCCCTATTTTACTAAAGATGGTGTAA
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   minnad15 1714 GATTATGGTACGAGTGTCAATTTGGGTTATACCGAGCCCTATTTTACTAAAGATGGTGTAA
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   eagand15 1754 GATTATGGTACGAGTGTCAATTTGGGTTATACCGAGCCCTATTTTACTAAAGATGGTGTAA
                 |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
     pakd15 1770 GATTATGGTACGAGTGTCAATTTGGGTTATACCGAaCCCTATTTTACTAAAGATGGTGTAA
                 |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
    sb33d15 1766 GATTATGGTACGAGTGTCAATTTGGGTTATACCGAgCCCTATTTTACTAAAGATGGTGTAA

consensus        GATTATGGTACGAGTGTCAATTTGGGTTATACCGAgCCCTATTTTACTAAAGATGGTGTAA
```

FIG.1F.(CONTINUED)

```
     cad15  1516 GTCTTGGTGGAAATGTTTTCTTTGAAAACTACGATAACTCTAAAAGTGATACATCCTCTAA
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   minnad15  1775 GTCTTGGTGGAAATGTTTTCTTTGAAAACTACGATAACTCTAAAAGTGATACATCCTCTAA
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    eagand15  1815 GTCTTGGTGGAAATGTTTTCTTTGAAAACTACGATAACTCTAAAAGTGATACATCCTCTAA
                 |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
      pakd15  1831 GTCTTGGTGGAAATaTTTTCTTTGAAAACTACGATAACTCTAAAAGTGATACATCCTCTAA
                 |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
     sb33d15  1827 GTCTTGGTGGAAATgTTTTCTTTGAAAACTACGATAACTCTAAAAGTGATACATCCTCTAA

consensus          GTCTTGGTGGAAATgTTTTCTTTGAAAACTACGATAACTCTAAAAGTGATACATCCTCTAA

     cad15  1577 CTATAAGCGTACGACTTACGGAAGTAATGTTACTTTAGGTTTCCCTGTAAATGAAAATAAC
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   minnad15  1836 CTATAAGCGTACGACTTACGGAAGTAATGTTACTTTAGGTTTCCCTGTAAATGAAAATAAC
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    eagand15  1876 CTATAAGCGTACGACTTACGGAAGTAATGTTACTTTAGGTTTCCCTGTAAATGAAAATAAC
                 |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
      pakd15  1892 CTATAAGCGTACGACTTATGGAAGTAATGTTACTTTAGGTTTCCCTGTAAATGAAAATAAC
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     sb33d15  1888 CTATAAGCGTACGACTTATGGAAGTAATGTTACTTTAGGTTTCCCTGTAAATGAAAATAAC

consensus          CTATAAGCGTACGACTTAcGGAAGTAATGTTACTTTAGGTTTCCCTGTAAATGAAAATAAC
```

FIG.1F.(CONTINUED)

```
     cad15 1638  TCCTATTATGTAGGATTAGGTCATACCTATAATAAAATTAGTAACTTTGCTCTAGAATATA
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   minnad15 1897 TCCTATTATGTAGGATTAGGTCATACCTATAATAAAATTAGTAACTTTGCTCTAGAATATA
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   eagand15 1937 TCCTATTATGTAGGATTAGGTCATACCTATAATAAAATTAGTAACTTTGCTCTAGAATATA
                 |||||||||||||||||||  ||||||||||||||||||||||||||||||||||||||
     pakd15 1953 TCCTATTATGTAGGATTAGGCCATACCTATAATAAAATTAGTAACTTTGCTCTAGAATATA
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    sb33d15 1949 TCCTATTATGTAGGATTAGGCCATACCTATAATAAAATTAGTAACTTTGCTCTAGAATATA

consensus        TCCTATTATGTAGGATTAGGtCATACCTATAATAAAATTAGTAACTTTGCTCTAGAATATA

     cad15 1699  ACCGTAATTTATATATTCAATCAATGAAATTTAAAGGTAATGGCATTAAAACAAATGACTT
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   minnad15 1958 ACCGTAATTTATATATTCAATCAATGAAATTTAAAGGTAATGGCATTAAAACAAATGACTT
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   eagand15 1998 ACCGTAATTTATATATTCAATCAATGAAATTTAAAGGTAATGGCATTAAAACAAATGACTT
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     pakd15 2014 ACCGTAATTTATATATTCAATCAATGAAATTTAAAGGTAATGGCATTAAAACAAATGACTT
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    sb33d15 2010 ACCGTAATTTATATATTCAATCAATGAAATTTAAAGGTAATGGCATTAAAACAAATGACTT

consensus        ACCGTAATTTATATATTCAATCAATGAAATTTAAAGGTAATGGCATTAAAACAAATGACTT
```

FIG.1F.(CONTINUED)

```
cad15  1760  TGATTTTTTCTTTTGGTTGGAACTATAACAGCCTTAATAGAGGCTATTTCCCAACTAAAGGG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15 2019 TGATTTTTTCTTTTGGTTGGAACTATAACAGCCTTAATAGAGGCTATTTCCCAACTAAAGGG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15 2059 TGATTTTTTCTTTTGGTTGGAACTATAACAGCCTTAATAGAGGCTATTTCCCAACTAAAGGG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15  2075  TGATTTTTTCTTTTGGTTGGAACTATAACAGCCTTAATAGAGGCTATTTCCCAACTAAAGGG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15 2071  TGATTTTTTCTTTTGGTTGGAACTATAACAGCCTTAATAGAGGCTATTTCCCAACTAAAGGG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
consensus     TGATTTTTTCTTTTGGTTGGAACTATAACAGCCTTAATAGAGGCTATTTCCCAACTAAAGGG

cad15  1821  GTTAAAGCAAGTCTTGGTGGACGAGTTACTATTCCAGGTTCTGATAACAAATACTACAAAC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15 2080 GTTAAAGCAAGTCTTGGTGGACGAGTTACTATTCCAGGTTCTGATAACAAATACTACAAAC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15 2120 GTTAAAGCAAGTCTTGGTGGACGAGTTACTATTCCAGGTTCTGATAACAAATACTACAAAC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pakd15  2136  GTTAAAGCAAGTCTTGGTGGACGAGTTACTATTCCAGGTTCTGATAACAAATACTACAAAC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sb33d15 2132  GTTAAAGCAAGTCTTGGTGGACGAGTTACaATTCCAGGTTCTGATAACAAATACTACAAAC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
consensus     GTTAAAGCAAGTCTTGGTGGACGAGTTACtATTCCAGGTTCTGATAACAAATACTACAAAC
```

FIG.1F.(CONTINUED)

```
     cad15  1882  TAAGTGCAGATGTACAGGGTTTCTACCCATTAGACAGAGATCACCTCTGGGTTGTATCTGC
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   minnad15  2141  TAAGTGCAGATGTACAGGGTTTCTACCCATTAGACAGAGATCACCTCTGGGTTGTATCTGC
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   eagand15  2181  TAAGTGCAGATGTACAGGGTTTCTACCCATTAGACAGAGATCACCTCTGGGTTGTATCTGC
                  |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     pakd15  2197  TAAGTGCAGATGTACAGGGTTTCTACCCATTAGACAGAGATCACCgCTGGGTTGTATCTGC
                  |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    sb33d15  2193  TAAGTGCAGATGTACAGGGTTTCTACCCATTAGACAGAGATCACCtCTGGGTTGTATCTGC

consensus          TAAGTGCAGATGTACAGGGTTTCTACCCATTAGACAGAGATCACCtCTGGGTTGTATCTGC

     cad15  1943  AAAAGCATCTGCAGGATATGCAAATGGTTTTGGAAACAAGCGTTTACCGTTCTATCAAACT
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   minnad15  2202  AAAAGCATCTGCAGGATATGCAAATGGTTTTGGAAACAAGCGTTTACCGTTCTATCAAACT
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   eagand15  2242  AAAAGCATCTGCAGGATATGCAAATGGTTTTGGAAACAAGCGTTTACCGTTCTATCAAACT
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     pakd15  2258  AAAAGCATCTGCAGGATATGCAAATGGTTTTGGAAACAAGCGTTTACCGTTCTATCAAACT
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    sb33d15  2254  AAAAGCATCTGCAGGATATGCAAATGGTTTTGGAAACAAGCGTTTACCGTTCTATCAAACT
```

FIG.1F.(CONTINUED)

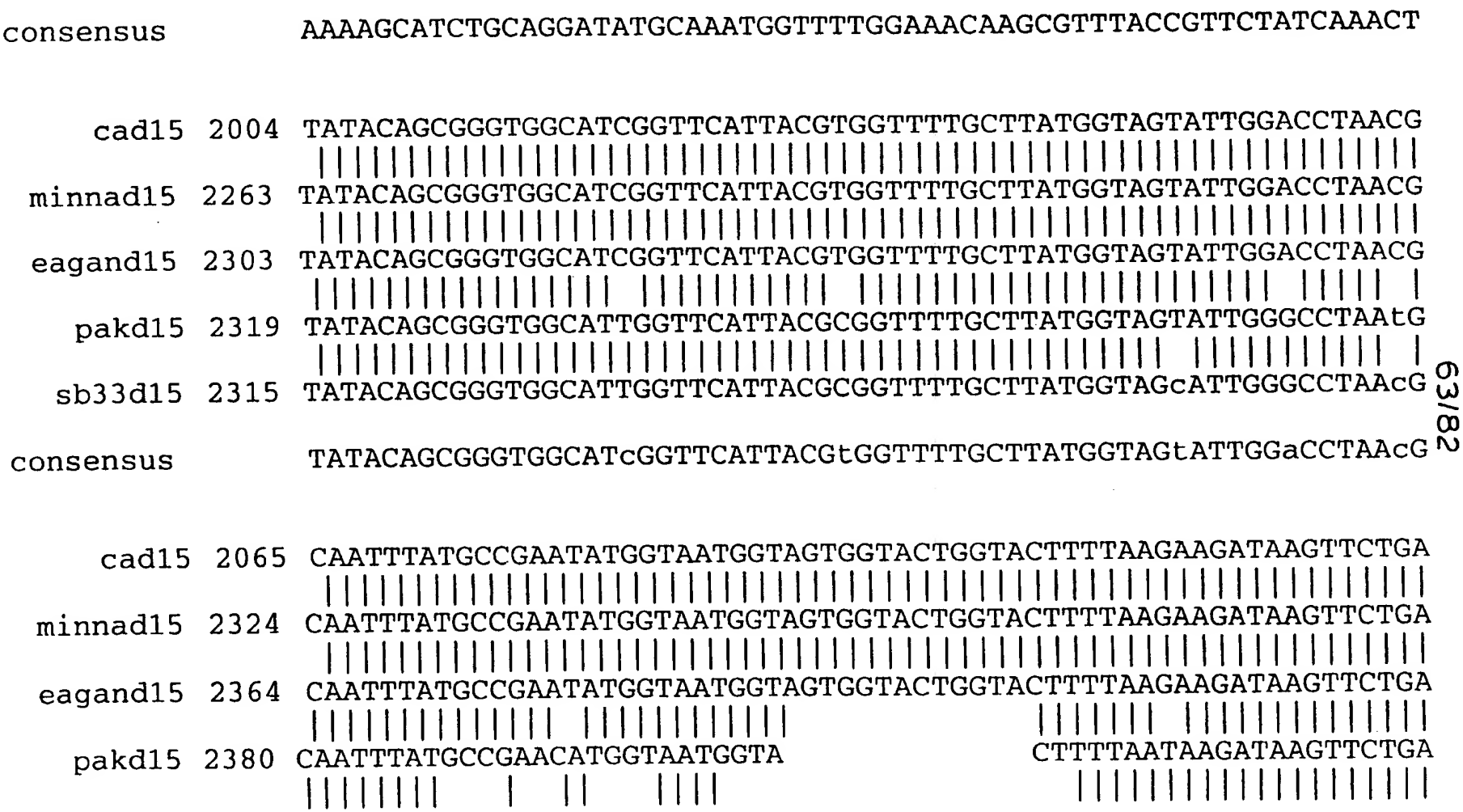

FIG.1F.(CONTINUED)

```
   sb33d15 2376 CAATTTATcaaGgtCAaaaTAAT                    aaaTTTAATAAGATAAGTTCTGA

consensus       CAATTTATgccGaatAtggTAATggtagtggtactggtactTTTAAgAAGATAAGTTCTGA


     cad15 2126 TGTGATTGGTGGTAATGCAATCGCTACAGCTAGCGCAGAGTTAATTGTGCCAACTCCATTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  minnad15 2385 TGTGATTGGTGGTAATGCAATCGCTACAGCTAGCGCAGAGTTAATTGTGCCAACTCCATTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  eagand15 2425 TGTGATTGGTGGTAATGCAATCGCTACAGCTAGCGCAGAGTTAATTGTGCCAACTCCATTT
                |||||||||||||||||||||| | || || || |||||  | ||||| ||||||||||||
    pakd15 2429 TGTGATTGGTGGTAATGCAATCaCaACtGCgAGtGCAGAacTtATTGTaCCAACTCCATTT
                |||||||||||||||||||||| | || || || |||||  | ||||| ||||||||||||
    sb33d15 2422 TGTGATTGGTGGTAATGCAATCgCtACaGCtAGcGCAGAgtTaATTGTgCCAACTCCATTT

consensus       TGTGATTGGTGGTAATGCAATCgCtACaGCtAGcGCAGAgtTaATTGTgCCAACTCCATTT


     cad15 2187 GTGAGCGATAAGAGCCAAAATACGGTCCGAACCTCCTTATTTGTTGATGCGGCAAGTGTTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  minnad15 2446 GTGAGCGATAAGAGCCAAAATACGGTCCGAACCTCCTTATTTGTTGATGCGGCAAGTGTTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  eagand15 2486 GTGAGCGATAAGAGCCAAAATACGGTCCGAACCTCCTTATTTGTTGATGCGGCAAGTGTTT
                ||||| ||||| |||||||||| |||||||||| |||||||||||||||||||||||||
```

FIG.1F.(CONTINUED)

```
pakd15      2490  GTGAGTGATAAaAGCCAAAATACAGTCCGAACCTCCCTATTTGTTGATGCGGCAAGTGTTT
                  ||||||||||| || ||||||||||||||||||||||||||||||||||||||||||||
sb33d15     2483  GTGAGTGATAAgAGtCAAAATACAGTCCGAACCTCCCTATTTGTTGATGCGGCAAGTGTTT

consensus         GTGAGcGATAAgAGcCAAAATACgGTCCGAACCTCCtTATTTGTTGATGCGGCAAGTGTTT


cad15       2248  GGAATACTAAATGGAAATCAGATAAAAATGGATTAGAGAGCGATGTATTAAAAAGATTGCC
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15    2507  GGAATACTAAATGGAAATCAGATAAAAATGGATTAGAGAGCGATGTATTAAAAAGATTGCC
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15    2547  GGAATACTAAATGGAAATCAGATAAAAATGGATTAGAGAGCGATGTATTAAAAAGATTGCC
                  |||||||||||||||||||||||||||||||||||| | || || ||| || ||
pakd15      2551  GGAATACTAAATGGAAATCAGATAAAAATGGATTAGAGAGCAAgGTCTTGAAAGACTTACC
                  |||||||||||||||||||||||||||||||||||| ||||||||||||||||||
sb33d15     2544  GGAATACTAAATGGAAATCAGATAAAAATGGATTAGAGAGCAAtGTCTTGAAAGACTTACC

consensus         GGAATACTAAATGGAAATCAGATAAAAATGGATTAGAGAGCgAtGTaTTaAAAagaTTgCC


cad15       2309  TGATTATGGCAAATCAAGCCGTATTCGCGCCTCTACAGGTGTCGGATTCCAATGGCAATCT
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15    2568  TGATTATGGCAAATCAAGCCGTATTCGCGCCTCTACAGGTGTCGGATTCCAATGGCAATCT
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

SUBSTITUTE SHEET

FIG.1F.(CONTINUED)

```
eagand15  2608  TGATTATGGCAAATCAAGCCGTATTCGCGCCTCTACAGGTGTCGGATTCCAATGGCAATCT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  pakd15  2612  TGATTATGGCAAATCAAGCCGTATTCGCGCCTCTACAGGTGTCGGATTCCAATGGCAATCT
                |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
  sb33d15 2605  cGATTATGGCAAATCAAGCCGTAcTCGCGCCTCTACAGGTGTCGGATTCCAATGGCAATCT

consensus       tGATTATGGCAAATCAAGCCGTAtTCGCGCCTCTACAGGTGTCGGATTCCAATGGCAATCT


   cad15  2370  CCTATTGGGCCATTGGTATTCTCTTATGCCAAACCAATTAAAAAATATGAAAATGATGATG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15  2629  CCTATTGGGCCATTGGTATTCTCTTATGCCAAACCAATTAAAAAATATGAAAATGATGATG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15  2669  CCTATTGGGCCATTGGTATTCTCTTATGCCAAACCAATTAAAAAATATGAAAATGATGATG
                |||||||| |||||||||| |||||||| ||||||||||||||||||||||||||||
  pakd15  2673  CCTATTGGACCATTGGTATTTTCTTATGCTAAACCAATTAAAAAATATGAAAATGATGATG
                |||| ||||||| ||||||||||||||||||||||||||||||||||||||||||||
  sb33d15 2666  CCTAgTGGACCAgTGGTATTTTCTTATGCTAAACCAATTAAAAAATATGAAAATGATGATG

consensus       CCTAtTGGgCCAtTGGTATTcTCTTATGCcAAACCAATTAAAAAATATGAAAATGATGATG


   cad15  2431  TCGAACAGTTCCAATTTAGTATTGGAGGTTCTTTCTAATAAATTGAACTTTTTTCTTCATC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

FIG.1F.(CONTINUED)

```
minnad15  2690  TCGAACAGTTCCAATTTAGTATTGGAGGTTCTTTCTAATAAATTGAACTTTTTTCTTCATC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15  2730  TCGAACAGTTCCAATTTAGTATTGGAGGTTCTTTCTAATAAATTGAACTTTTTTCTTCATC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 pakd15   2734  TCGAACAGTTCCAATTTAGTATTGGGGGcTCTTTCTAATAAATTGAACTTTTTTCGTCATC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 sb33d15  2727  TCGAACAGTTCCAATTTAGTATTGGGGGtTCTTTCTAATAAATTGAACTTTTTTCGTCATC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
consensus       TCGAACAGTTCCAATTTAGTATTGGaGGtTCTTTCTAATAAATTGAACTTTTTTCtTCATC

 cad15    2492  AGAACTCAAAAACAACGTTCTCTGCCTAATTTAATTGGGCAGAGAAAATATTAAACCCATC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
minnad15  2751  AGAACTCAAAAACAACGTTCTCTGCCTAATTTAATTGGGCAGAGAAAATATTAAACCCATC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
eagand15  2791  AGAACTCAAAAACAACGTTCTCTGCCTAATTTAATTGGGCAGAGAAAATATTAAACCCATC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 pakd15   2795  AGAACTCAAAAACgACGTTCTCTGCCTAATTgAATTGGGCAGAGAAAATATTAAACCCATC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 sb33d15  2788  AGAACTCAAAAACaACGTTCTCTGCCTAATTtAATTGGGCAGAGAAAATATTAAAaCCATC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
consensus       AGAACTCAAAAACaACGTTCTCTGCCTAATTtAATTGGGCAGAGAAAATATTAAAcCCATC
```

FIG.1F.(CONTINUED)

```
cad15    2553 ATTTAATTAAGGATATTTATCAAATGAAAAACATCGCAAAAGTAACCGCACTTGCTTTAGG
minnad15 2812 ATTTAATTAAGGATATTTATCAAATGAAAAACATCGCAAAAGTAACCGCACTTGCTTTAGG
eagand15 2852 ATTTAATTAAGGATATTTATCAAATGAAAAACATCGCAAAAGTAACCGCACTTGCTTTAGG
pakd15   2856 ATTTAATTAAGGATATTTATCAAATGAAAAACATCGCAAAAGTAACCGCACTTGCTTTAGG
sb33d15  2849 ATTTAATTAAGGATATTTATCAAATGAAAAACATCGCcAAAGTAACCGCACTTGCTTTAGG

consensus     ATTTAATTAAGGATATTTATCAAATGAAAAACATCGCaAAAGTAACCGCACTTGCTTTAGG

cad15    2614 TATTGCACTTGCTTCAGGCTATGCTTCCGCTGAAGAAAAAATTGCTTTCATTAATGCaGGT
minnad15 2873 TATTGCACTTGCTTCAGGCTATGCTTCCGCTGAAGAAAAAATTGCTTTCATTAATGCgGGT
eagand15 2913 TATTGCACTTGCTTCAGGCTATGCTTCCGCTGAAGAAAAAATTGCTTTCATTAATGC AcT
pakd15   2917 TtTTGCACTTGCTTCAGGCTATGCTTCCGCTGAAGAAAAAATTGCTTTCATTAATGC AGG
sb33d15  2910 TaTTGCACTTGCTTCAGGCTATGCTgCaGCTGAAGAAAAAATTGCTTTtATTAATGC AGG

consensus     TaTTGCACTTGCTTCAGGCTATGCTtCcGCTGAAGAAAAAATTGCTTTcATTAATGC-agt
```

FIG.1F.(CONTINUED)

```
     cad15 2675 atattTTTcaAcatCacccagatcgccaagcggtagcagataaacttgatgctgaatttaa
                      |||   |    |
 minnad15 2934 TATAnTTTnCAAggCnaagg
                |||| ||| |||
 eagand15 2973 TATAtTTTTCAA
                |    ||||  |
   pakd15 2977 TTATATTTTtcAa
                |||||
  sb33d15 2970 TTATA

consensus       ttat-ttttcaaa-c-----gatcgccaagcggtagcagataaacttgatgctgaatttaa


     cad15 2736 acctgtagctgagaaattagcagcaagcaaaaaagaagttgatgataaaattgctgctgct

 minnad15 2954

 eagand15 2985

   pakd15 2990

  sb33d15 2975

consensus       acctgtagctgagaaattagcagcaagcaaaaaagaagttgatgataaaattgctgctgct
```

FIG.1F.(CONTINUED)

```
cad15 2797  cgtaaaaaagtagaagcaaaagttgcggctttagaaaaagatgcacctcgcttacgtcaag

minnad15 2954

eagand15 2985

pakd15 2990

sb33d15 2975

consensus        cgtaaaaaagtagaagcaaaagttgcggctttagaaaaagatgcacctcgcttacgtcaag

cad15 2858  ctgatattcaaaaacgccaacaggagattaataaattaggtgcggctgaagatgctgaatt

minnad15 2954

eagand15 2985

pakd15 2990

sb33d15 2975

consensus        ctgatattcaaaaacgccaacaggagattaataaattaggtgcggctgaagatgctgaatt
```

FIG.1F.(CONTINUED)

```
cad15  2919  acaaaaattaatgcaagaacaagataaaaaa

minnad15  2954

eagand15  2985

pakd15  2990

sb33d15  2975

consensus        acaaaaattaatgcaagaacaagataaaaaa
```

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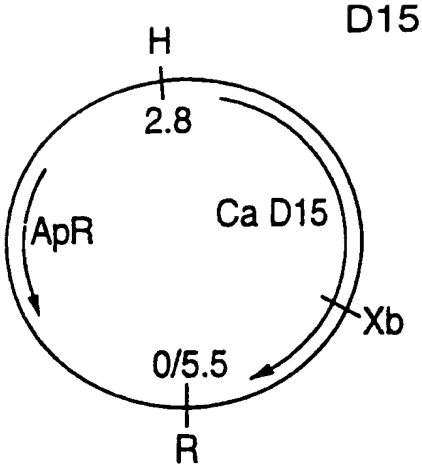
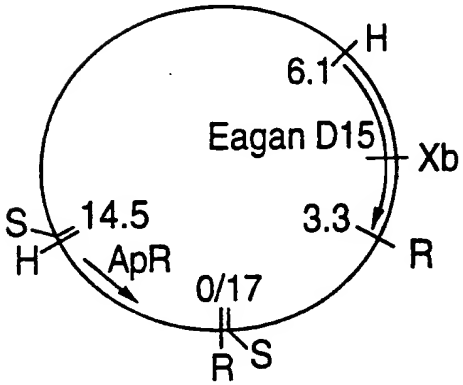
D15 CLONES



pUC19/D15



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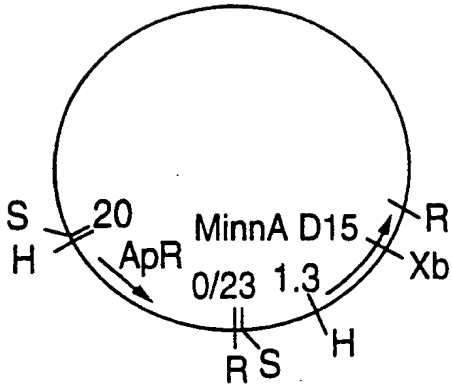
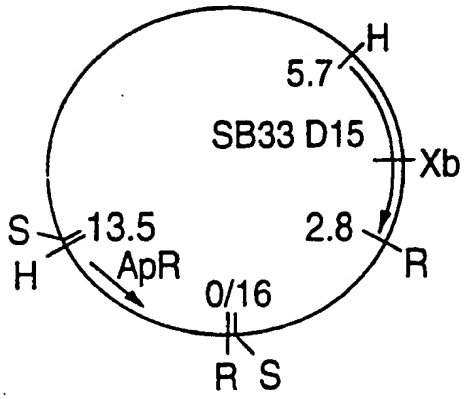


DS-691-1-5



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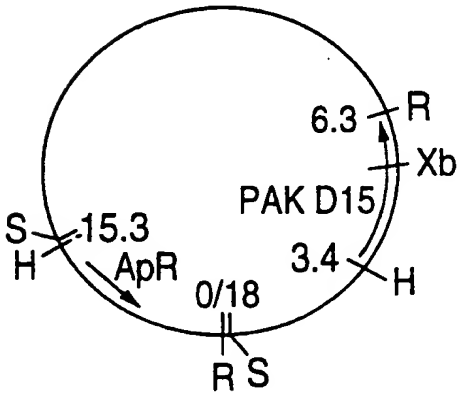


JB-1042-9-4

FIG.2.

D15 SEQUENCE COMPARISON

```
MKKLLIASLLFGITTTVFAAPFVAKDIRVDGVQGDLEQQIRASLPVRAGQRVTDNDVANIVRSLFVSGRFDDVKAHQEGDVLVVSVVAKSIISDVKIKGN  Ca
.....  Eagan
.....  MinnA
.....  SB33
.....  PAK

SVIPTEALKQNLDANGFKVGDVLIREKLNEFAKSVKEHYASVGRYNATVEPIVNILPNNRAEILIQINEDDKAKLASLTFKGNESVSSSTLQEQMELQPD  Ca
.....  Eagan
.....  MinnA
.I.....I.....  SB33
.....  PAK

SWWKLWGNKFEGAQFEKDLQSIRDYYLNNGYAKAQITKTDVQLNDEKTKVNVTIDVNEGLQYDLRSARIIGNLGGMSAELEPLLSALHLNDTFRRSDIAD  Ca
.....  Eagan
.....  MinnA
.....A.....  SB33
.....A.....  PAK

VENAIKAKLGERGYGSAIVNSVPDFDDANKTLAITLVVDAGRRLTVRQLRFEGNIVSADSTLRQEMRQQEGIWYNSQLVELGKIRLDRTGFFETVENRID  Ca
.....  Eagan
.....  MinnA
.....NT.....F.....H.....  SB33
.....NT.....F.....  PAK
```

FIG.3A.

```
PINGSNDEVDVVYKVKERNTGSINFGIGYGIESGISYQASVKQDNFLGIGAAVSIAGTKNDYGTSVNLGYTEPYFTKDGVSLGGNVFFENYDNSKSDTSS  Ca
.....................................................................................................  Eagan
.....................................................................................................  MinnA
.....................................................................................................  SB33
.....................T.I.............................................I.....................................  PAK

NYKRTTYGSNVTLGFPVNENNSYYVGLGHTYNKISNFALEYNRNLYTQSMKFKGNGIKTNDFDSFGMNYNSLNFKGYFPTKGVKASLGGRVTTRGSDNKY  Ca
.....................................................................................................  Eagan
.....................................................................................................  MinnA
.....................................................................................................  SB33
.....................................................................................................  PAK

YKLSADVQGFYPLDRDHLWVSAKASAGYANGFGNKRLPFYQTYTAGGIGSLRGFAYGSIGPNAIYAEYGNGSGIGTFKKISSDVIGGNAIATASAELIV  Ca
.....................................................................................................  Eagan
.....................................................................................................  MinnA
.....................................................--Q.QNNK---N.....................................  SB33
.....R...............................................H..---...N.....................T.....  PAK

PTPFVSDKSQNTVRISLFVDAASWNTKWKSDKNGLESDVLKRLPDYGKSSRIRASTGVGFQMQSPIGPLVFSYAKPIKKYENDDVEQFQFSIGGSF**  Ca
.....................................................................................................**  Eagan
.....................................................................................................**  MinnA
.....................N..D.....................................................................**  SB33
.....................K..D.....................................................................**  PAK
```

FIG.3B.

Construction of plasmid expressing SB33 D15

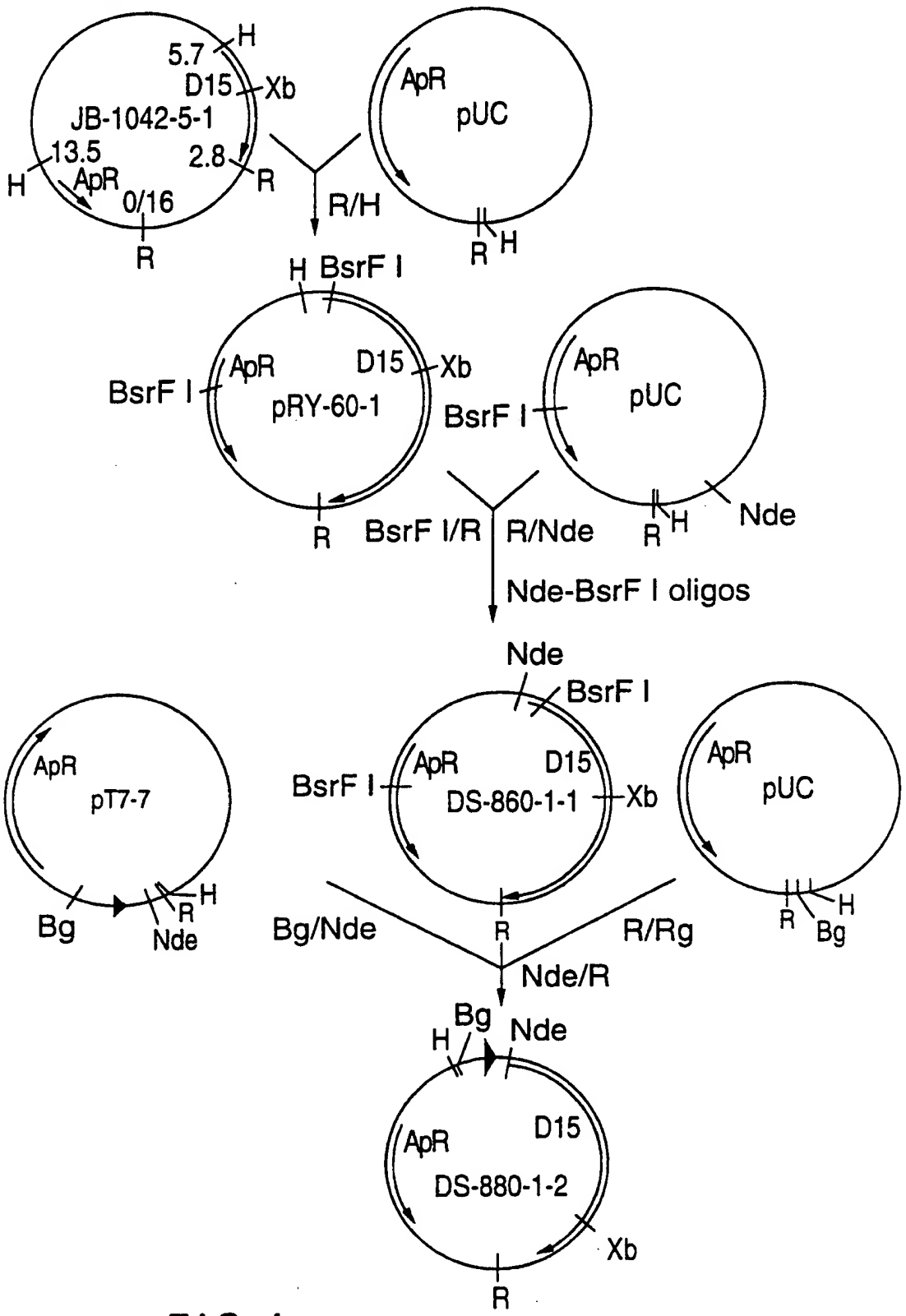
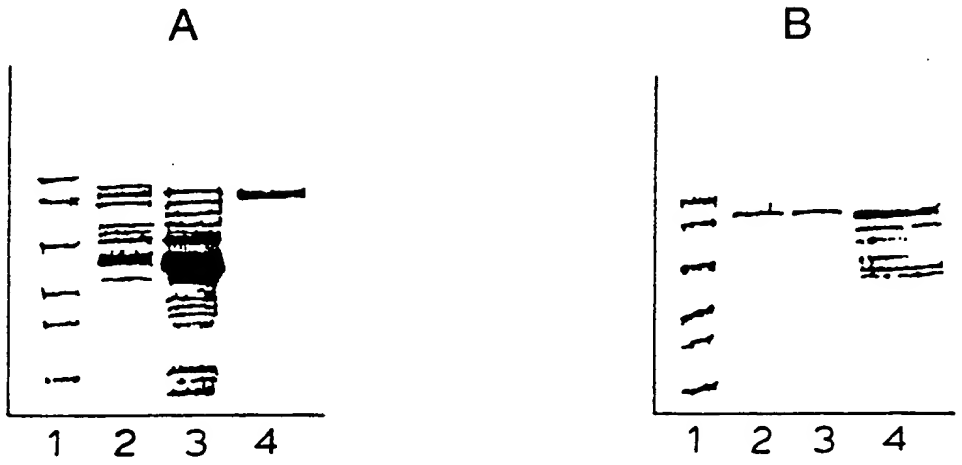


FIG.4.

SUBSTITUTE SHEET

PURIFICATION OF D15 FROM A NON-TYPEABLE HAEMOPHILUS INFLUENZAE STRAIN 30

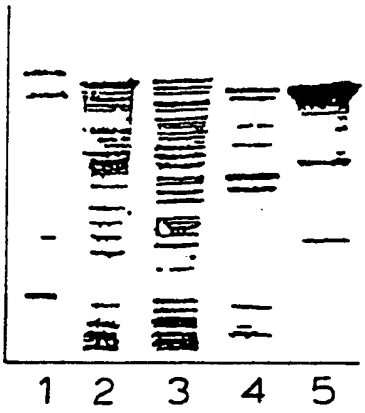


PROTEIN STAIN WESTERN BLOT

1. Low MW markers
2. Strain 30
3. Native D15 crude extract
4. D15 after anti-D15 affinity chromatography

FIG.5.

PURIFICATION OF FULL LENGTH RECOMBINANT D15



1. Protein M.W. Markers
2. Lysate of E. coli expressed rD15
3. Soluble protein in Tris-HC1 buffer extract
4. Soluble proteins in Tris/Triton X-100/ EDTA extraction buffer
5. rD15 inclusion bodies

FIG.6.

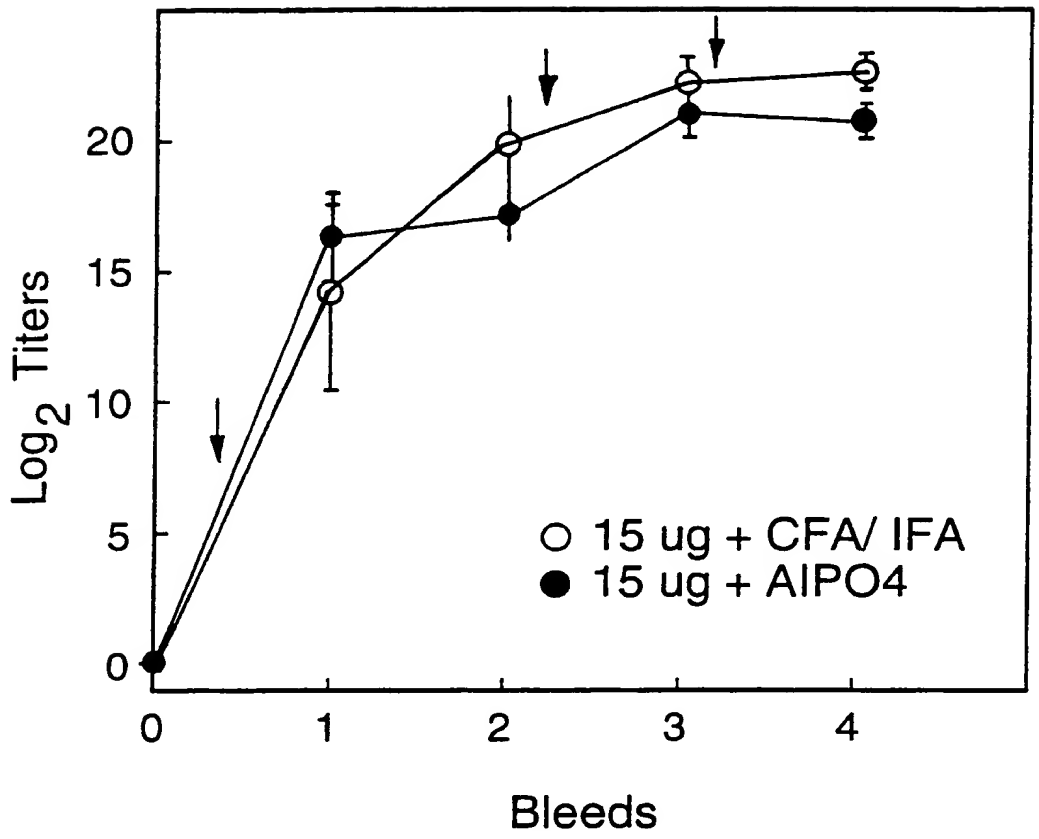


FIG.7.

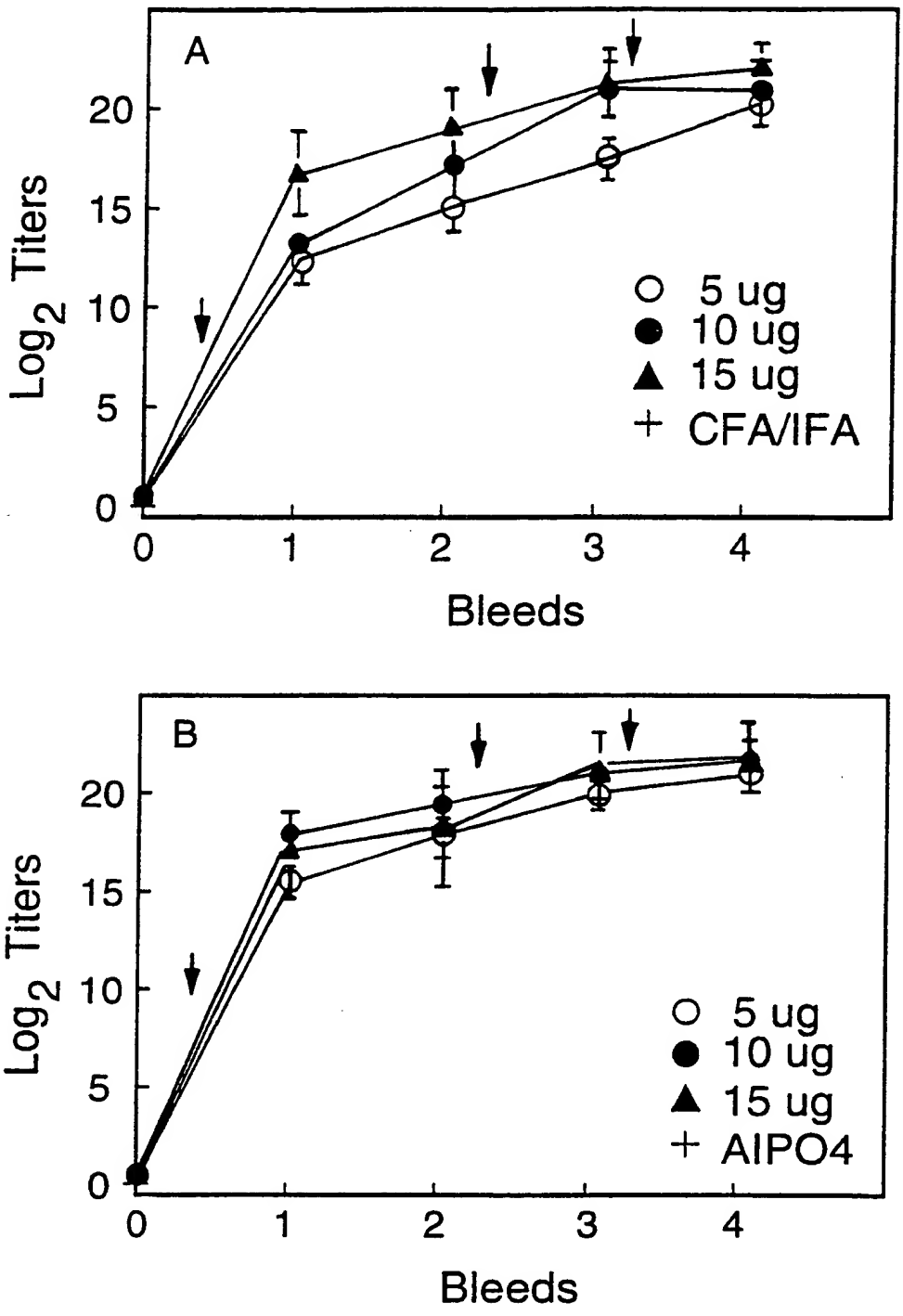
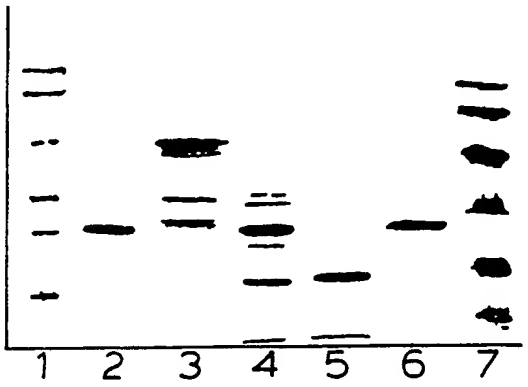


FIG.8.

PURIFICATION OF TRUNCATED D15 FROM D15-GST FUSION PROTEIN



1. Prestain low MW markers
2. GST standard
3. GST-(D15 fragment) fusion protein
4. Fusion protein cleaved by thrombin
5. rD15 fragment
6. GST
7. Low MW markers

FIG.9.

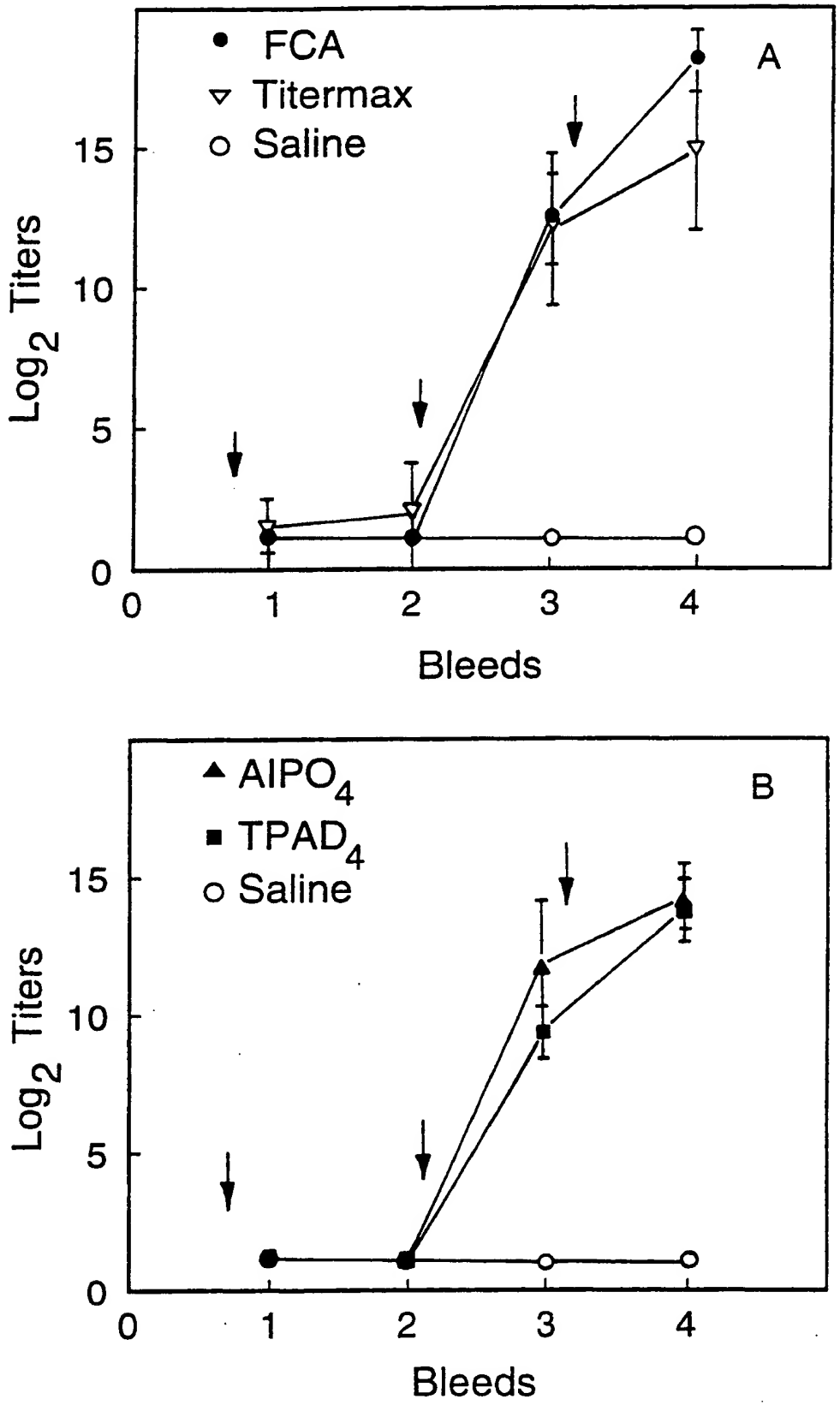
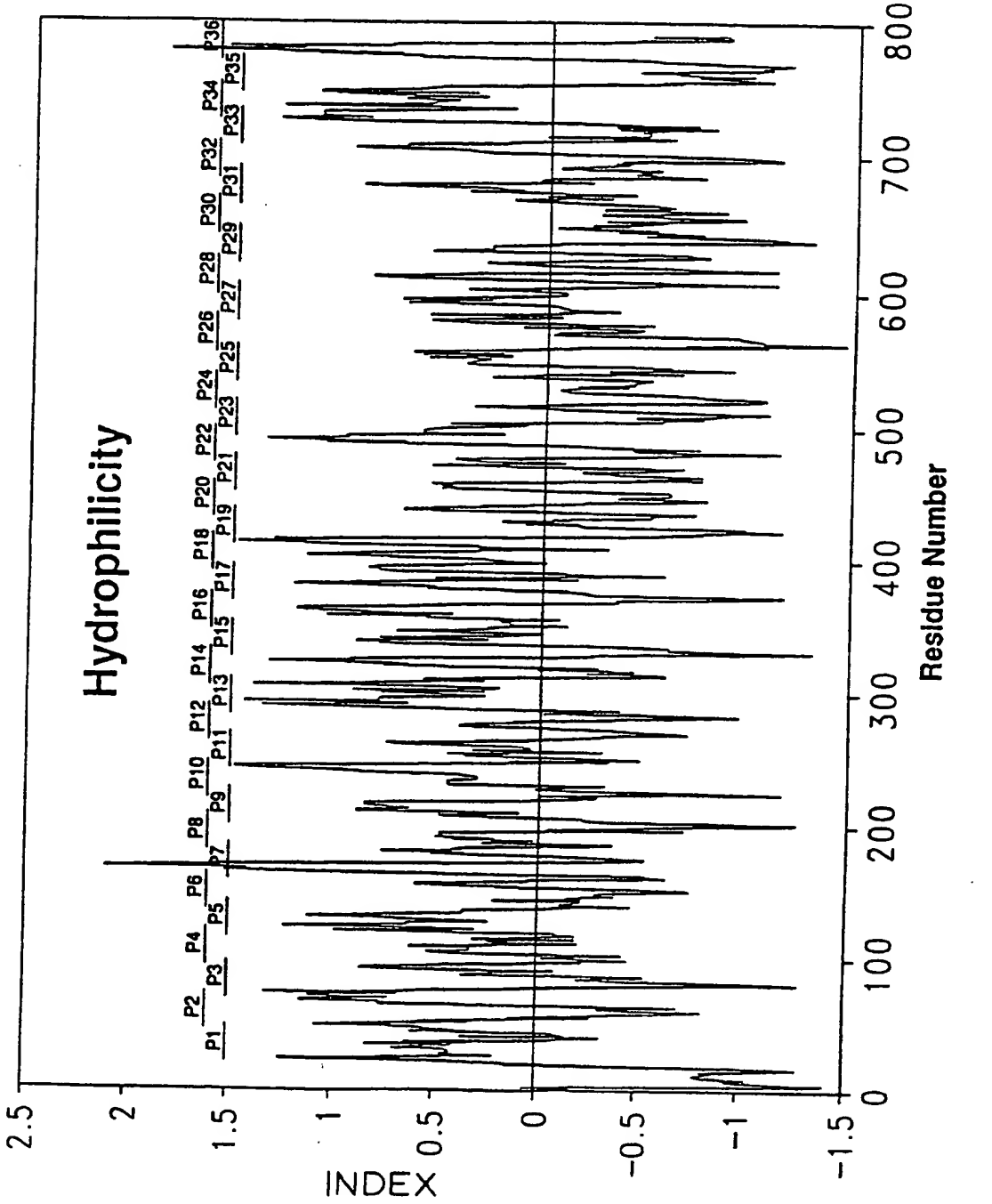


FIG.10.

FIGURE 11



INTERNATIONAL SEARCH REPORT

In ional Application No
PCT/CA 93/00501

A. CLASSIFICATION OF SUBJECT MATTER

C 12 N 15/31,C 07 K 13/00,A 61 K 39/102,
//(C 12 N 15/31; C 12 R 1/21)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A 61 K,C 07 K,C 12 N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	INFECTION AND IMMUNITY, vol. 58, no. 4, April 1990 W.R. THOMAS et al. "Expression in Escherichia coli of a High-Molecular-Weight Protective Surface Antigen Found in Nontypeable and Type b Haemophilus influenzae" pages 1909-1913, the whole document. --	1,4,6, 27
A	EP, A2, 0 378 929 (CONNAUGHT LABORATORIES LIMITED) 25 July 1990 (25.07.90), claims. --	1,4,6, 12,16, 20,22
A	WO, A1, 91/06 652	1,4,6,

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 1994	25 -03- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	WOLF e.h.

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INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/CA 93/00501

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	(CONNAUGHT LABORATORIES LIMITED et al.) 16 May 1991 (16.05.91), claims.	12,16, 20,22
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A	EP, A1, 0 281 673 (THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK) 14 September 1988 (14.09.88), claims.	1,4,6, 12,18, 22,27
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A	US, A, 5 013 664 (BRODEUR et al.) 07 May 1991 (07.05.91), abstract.	1,27

ANHANG

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

ANNEX

to the International Search Report to the International Patent Application No.

ANNEXE

au rapport de recherche international relatif à la demande de brevet international n°

PCT/CA 93/00501 SAE 82214

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

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Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
EP A2 378929	25-07-90	GB A0 8902178	22-03-89
		CA AA 2006587	22-06-90
		CA AA 2006587	23-06-90
		EP A3 378929	01-08-90
		JP A2 2283286	20-11-90
		GB A0 8830124	22-02-89
WO A1 9106652	16-05-91	CA AA 2072095	01-05-91
		EP A1 500576	02-09-92
		GB A0 8924473	20-12-89
		JP T2 5501202	11-03-93
EP A1 281673	14-09-88	US A 5173294	22-12-92
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		DK A 6020/87	19-05-88
		JP A2 1157387	20-06-89
US A 5013664	07-05-91	keine - none - rien	